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(54) Title: **HUMAN WNT GENE**

(57) Abstract: The Wnt proteins are a family of secreted glycoproteins, which, in many organisms, have a role in the morphological development of tissues in both embryonic and adult contexts. The present invention provides a new form of human Wnt, designated "Zwnt3."

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HUMAN WNT GENE

TECHNICAL FIELD

10 The present invention relates generally to a new gene that encodes a cellular signaling molecule. In particular, the present invention relates to a novel Wnt, designated "Zwnt3," and to nucleic acid molecules encoding Zwnt3.

15 BACKGROUND OF THE INVENTION

Cellular differentiation of multicellular organisms is controlled by hormones and polypeptide growth factors. These diffusable molecules allow cells to communicate with each other, to act in concert to form tissues and organs, and to repair and regenerate damaged tissue. Examples of hormones and growth factors include the
20 steroid hormones, parathyroid hormone, follicle stimulating hormone, the interferons, the interleukins, platelet derived growth factor, epidermal growth factor, and granulocyte-macrophage colony stimulating factor, among others.

Hormones and growth factors influence cellular metabolism by binding to receptor proteins. Certain receptors are integral membrane proteins that bind with
25 the hormone or growth factor outside the cell, and that are linked to signaling pathways within the cell, such as second messenger systems. Other classes of receptors are soluble intracellular molecules.

Wnt proteins are emerging as one of the pre-eminent families of signaling molecules in animal development. To date, murine *Wnt* genes include *Wnt-1*,
30 *Wnt-2*, *Wnt-2B/13*, *Wnt-3*, *Wnt-3A*, *Wnt-4*, *Wnt-5A*, *Wnt-5B*, *Wnt-6*, *Wnt-7A*, *Wnt-7B*, *Wnt-8A*, *Wnt-8B*, *Wnt-10A*, *Wnt-10B*, *Wnt-11*, and *Wnt-15*, while the following human *Wnt* genes have been described: *Wnt-1*, *Wnt-2*, *Wnt-2B/13*, *Wnt-3*, *Wnt-4*, *Wnt-5A*, *Wnt-7A*, *Wnt-8A*, *Wnt-8B*, *Wnt-10B*, *Wnt-11*, *Wnt-14*, and *Wnt-15*. See, for example, Nusse and Varmus, *Cell* 31:99 (1982), van Ooyen *et al.*, *EMBO J.* 4:2905 (1985), Wainwright
35 *et al.*, *EMBO J.* 7:1743 (1988), McMahon and McMahon, *Development* 107:643 (1989), Gavin *et al.*, *Genes Dev.* 4:2319 (1990), Roelink *et al.*, *Proc. Nat'l Acad. Sci. USA* 87:4519 (1990), Roelink and Nusse, *Genes Dev.* 5:381 (1991), Clark *et al.*, *Genomics* 18:249 (1993), Roelink *et al.*, *Genomics* 17:790 (1993), Adamson *et al.*,

Genomics 24:9 (1994), Huguet *et al.*, *Cancer Res.* 54:2615 (1994), Bouillet, *Mech. Dev.* 58:141 (1996), Ikegawa *et al.*, *Cytogenet. Cell Genet.* 74:149 (1996), Katoh *et al.*, *Oncogene* 13:873 (1996), Lako *et al.*, *Genomics* 35:386 (1996), Wang and Shackleford, *Oncogene* 13:1537 (1996), Bergstein, *Genomics* 46:450 (1997), Bui *et al.*, *Oncogene* 5 14:1249 (1997), and Grove *et al.*, *Development* 125:2315 (1998).

Wnt genes typically encode secreted glycoproteins having 350-400 amino acids, and the proteins often include a conserved pattern of 23-24 cysteine residues in addition to other invariant residues (Cadigan and Nusse, *Genes & Dev.* 11:3286 (1997)). Following cellular secretion, *Wnt* proteins are believed to reside 10 mainly in the extracellular matrix or to associated with the cellular surface.

According to the classical *Wnt* signaling pathway model, *Wnt* proteins induce gene expression by de-repressing a signal pathway via a so-called "Frizzled" transmembrane receptor (see, for example, Brown and Moon, *Curr. Opin. Cell Biol.* 10:182 (1998)). In the absence of *Wnt*, glycogen synthase kinase-3 β activity results in 15 the degradation of the free cytosolic pool of β -catenin. The association of cognate *Wnt* proteins and Frizzled receptors leads to the activation of a signaling pathway. The most proximal intracellular component of this pathway is the Disheveled protein, which becomes phosphorylated and inhibits glycogen synthase kinase-3 β . Consequently, the pool of intracellular β -catenin increases, and β -catenin can interact with members of the 20 lymphoid enhancer/T cell factor (LEF/TCF) family of architectural transcription factors in the nucleus. These complexes bind consensus LEF/TCF sites in promoters and induce transcription of *Wnt*-responsive genes.

Several secreted factors inhibit *Wnt* signaling (see, for example, Finch *et al.*, *Proc. Nat'l Acad. Sci. USA* 94:6770 (1997); Moon *et al.*, *Cell* 88:725 (1997); 25 Luyten *et al.*, WO 98/16641); Brown and Moon, *Curr. Opin. Cell Biol.* 10:182 (1998)). The Frzb proteins, for example, bind to secreted *Wnt* proteins and prevent productive interactions between *Wnt* and Frizzled proteins. These proteins contain a region that is homologous to a putative *Wnt*-binding domain of Frizzled proteins.

The *Wnt* proteins are multipotent, and the proteins are capable of 30 inducing different biological responses in both embryonic and adult contexts (see, for example, Ingham, *TIG* 12:382 (1996)). This type of broad activity is shared with fibroblast growth factors, transforming growth factors β , and nerve growth factors (Nusse and Varmus, *Cell* 69:1073 (1992)). When over-expressed, *Wnt* proteins can promote tumor formation (Erdreich-Epstein and Shackleford, *Growth Factors* 15:149 35 (1998)). Knock-out mutations in mice have shown *Wnt* proteins to be essential for brain development, and the out growth of embryonic primordia for kidney, tail bud and limb bud (McMahon and Bradley, *Cell* 62:1073 (1990), Thomas and Capecchi, *Nature*

346:847 (1990), Stark *et al.*, *Nature* 372:679 (1994), Takada *et al.*, *Genes Dev.* 8:174 (1994), and Parr and McMahon, *Nature* 374:350 (1995)).

Although new uses of known Wnt proteins may be discovered, a need exists for the provision of new Wnt proteins for biopharmaceuticals.

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BRIEF SUMMARY OF THE INVENTION

The present invention provides a novel Wnt protein, designated "Zwnt3." The present invention also provides Zwnt3 variant polypeptides and Zwnt3
10 fusion proteins, as well as nucleic acid molecules encoding such polypeptides and proteins, and methods for using these nucleic acid molecules and amino acid sequences.

DETAILED DESCRIPTION OF THE INVENTION

15

1. Overview

The present invention provides nucleic acid molecules that encode a new human Wnt protein, designated as "Zwnt3." An illustrative nucleotide sequence that encodes Zwnt3 is provided by SEQ ID NO:1. The encoded polypeptide has the
20 following amino acid sequence: MGNLFMLWAA LGICCAAFSA SAWSVNNFLI TGPKEYLYTT TSVALGAQSG IEECKFQFAW ERWNCPENAL QLSTHNRLRS ATRETSFIHA ISSAGVMYII TKNCSMGDFE NCGCDGSNNG KTGHHGWIWG GCSDNVEFGE RISKLFVDSL EKGKDARALM NLHNNRAGRL AVRATMKRTC KCHGISGSCS IQTCWLQLAE FREMGDYKA KYDQALKIEM DKRQLRAGNS
25 AEGHWVPAEA FLPSAEAELI FLEESPDYCT CNSSLGIYGT EGRECLQNSH NTSRWERRSC GRLCTECGLQ VEERKTEVIS SCNCKFQWCC TVKCDQCRHV VSKYYCARSP GSAQSLELSV TPTNLPTWTL CQKQQEFGL YHRLPAKDS FQGNTASFRF VSYSPISLPF WFILNKLAI KVTEQ (SEQ ID NO:2). Thus, the *Zwnt3* gene described herein encodes a polypeptide of 415 amino acids, as shown in
30 SEQ ID NO:2. The *Zwnt3* gene is expressed in brain tissue.

As detailed below, the present invention provides isolated polypeptides having an amino acid sequence that is at least 70%, at least 80%, or at least 90% identical to the amino acid sequence of SEQ ID NO:2. Certain of these polypeptides can specifically bind with an antibody that specifically binds with a polypeptide
35 consisting of the amino acid sequence of SEQ ID NO:2, with the provision that such

polypeptides include neither murine Wnt-8D (GenBank Accession No. Z68889; SEQ ID NO:4) nor human Wnt-8B (GenBank Accession No. X91940; SEQ ID NO:6).

An illustrative polypeptide is a polypeptide that comprises the amino acid sequence of SEQ ID NO:2. Additional exemplary polypeptides include polypeptides comprising an amino acid sequence of at least 15 contiguous amino acids of an amino acid sequence selected from the group consisting of: amino acid residues 44 to 62 of SEQ ID NO:2, amino acid residues 69 to 97 of SEQ ID NO:2, amino acid residues 120 to 138 of SEQ ID NO:2, amino acid residues 143 to 177 of SEQ ID NO:2, amino acid residues 184 to 215 of SEQ ID NO:2, amino acid residues 217 to 231 of SEQ ID NO:2, amino acid residues 217 to 248 of SEQ ID NO:2, amino acid residues 231 to 248 of SEQ ID NO:2, amino acid residues 276 to 297 of SEQ ID NO:2, amino acid residues 321 to 345 of SEQ ID NO:2, amino acid residues 345 to 415 of SEQ ID NO:2, and amino acid residues 321 to 415 of SEQ ID NO:2.

The present invention further provides antibodies and antibody fragments that specifically bind with such polypeptides. Exemplary antibodies include polyclonal antibodies, murine monoclonal antibodies, humanized antibodies derived from murine monoclonal antibodies, and human monoclonal antibodies. Illustrative antibody fragments include $F(ab')_2$, $F(ab)_2$, Fab' , Fab , Fv , $scFv$, and minimal recognition units. The present invention also includes anti-idiotypic antibodies that specifically bind with such antibodies or antibody fragments. The present invention further includes compositions comprising a carrier and a peptide, polypeptide, antibody, or anti-idiotypic antibody described herein.

The present invention also provides isolated nucleic acid molecules that encode a *Zwnt3* polypeptide, wherein the nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:3, (b) a nucleic acid molecule encoding the amino acid sequence of SEQ ID NO:2, and (c) a nucleic acid molecule that remains hybridized following stringent wash conditions to a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1, or the complement of SEQ ID NO:1, with the provision that such nucleic acid molecules encode neither murine Wnt-8D (GenBank Accession No. Z68889; SEQ ID NO:4) nor human Wnt-8B (GenBank Accession No. X91940; SEQ ID NO:6).

Illustrative nucleic acid molecules include those in which any difference between the amino acid sequence encoded by the nucleic acid molecule and the corresponding amino acid sequence of SEQ ID NO:2 is due to a conservative amino acid substitution. The present invention further contemplates isolated nucleic acid molecules that comprise a nucleotide sequence of SEQ ID NO:1.

The present invention also includes vectors and expression vectors comprising such nucleic acid molecules. Such expression vectors may comprise a transcription promoter, and a transcription terminator, wherein the promoter is operably linked with the nucleic acid molecule, and wherein the nucleic acid molecule is operably linked with the transcription terminator. The present invention further includes recombinant host cells comprising these vectors and expression vectors. Illustrative host cells include bacterial, yeast, avian, fungal, insect, mammalian, and plant cells. Recombinant host cells comprising such expression vectors can be used to prepare Zwnt3 polypeptides by culturing such recombinant host cells that comprise the expression vector and that produce the Zwnt3 protein, and isolating the Zwnt3 protein from the cultured recombinant host cells. The present invention further includes products made by such processes.

The present invention also contemplates methods for detecting the presence of *Zwnt3* RNA in a biological sample, comprising the steps of (a) contacting a *Zwnt3* nucleic acid probe under hybridizing conditions with either (i) test RNA molecules isolated from the biological sample, or (ii) nucleic acid molecules synthesized from the isolated RNA molecules, wherein the probe has a nucleotide sequence comprising a portion of the nucleotide sequence of SEQ ID NO:1, or its complement, and (b) detecting the formation of hybrids of the nucleic acid probe and either the test RNA molecules or the synthesized nucleic acid molecules, wherein the presence of the hybrids indicates the presence of *Zwnt3* RNA in the biological sample. Additional examples of suitable probes include nucleic acid molecules comprising a portion of either nucleotides 694 to 741 of SEQ ID NO:1, or nucleotides 961 to 1245 of SEQ ID NO:1. An example of a biological sample is a human biological sample, such as a biopsy or autopsy specimen.

The present invention further provides methods for detecting the presence of Zwnt3 polypeptide in a biological sample, comprising the steps of: (a) contacting the biological sample with an antibody or an antibody fragment that specifically binds with a polypeptide having the amino acid sequence of SEQ ID NO:2, wherein the contacting is performed under conditions that allow the binding of the antibody or antibody fragment to the biological sample, and (b) detecting any of the bound antibody or bound antibody fragment. Such an antibody or antibody fragment may further comprise a detectable label selected from the group consisting of radioisotope, fluorescent label, chemiluminescent label, enzyme label, bioluminescent label, and colloidal gold. An exemplary biological sample is a human biological sample, such as a biopsy or autopsy specimen.

The present invention also provides kits for performing these detection methods. For example, a kit for detection of *Zwnt3* gene expression may comprise a container that comprises a nucleic acid molecule, wherein the nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, (b) a nucleic acid molecule comprising the complement of the nucleotide sequence of SEQ ID NO:1, (c) a nucleic acid molecule that is a fragment of (a) consisting of at least eight nucleotides, and (d) a nucleic acid molecule that is a fragment of (b) consisting of at least eight nucleotides. Such a kit may also comprise a second container that comprises one or more reagents capable of indicating the presence of the nucleic acid molecule. On the other hand, a kit for detection of *Zwnt3* protein may comprise a container that comprises an antibody, or an antibody fragment, that specifically binds with a polypeptide consisting of the amino acid sequence of SEQ ID NO:2.

The present invention also contemplates anti-idiotypic antibodies, or anti-idiotypic antibody fragments, that specifically bind an antibody or antibody fragment that specifically binds a polypeptide consisting of the amino acid sequence of SEQ ID NO:2.

The present invention further provides variant *Zwnt3* polypeptides, which comprise an amino acid sequence that shares an identity with the amino acid sequence of SEQ ID NO:2 selected from the group consisting of at least 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, or greater than 95% identity, and wherein any difference between the amino acid sequence of the variant polypeptide and the amino acid sequence of SEQ ID NO:2 is due to one or more conservative amino acid substitutions.

The present invention also provides fusion proteins comprising a *Zwnt3* polypeptide moiety. Such fusion proteins can further comprise an immunoglobulin moiety. In such fusion proteins, the immunoglobulin moiety may be an immunoglobulin heavy chain constant region, such as a human F_c fragment. The present invention further includes isolated nucleic acid molecules that encode such fusion proteins.

These and other aspects of the invention will become evident upon reference to the following detailed description. In addition, various references are identified below.

2. Definitions

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

5 As used herein, "nucleic acid" or "nucleic acid molecule" refers to polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-
10 occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (e.g., α -enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters.
15 Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs
20 of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. The term "nucleic acid molecule" also includes so-called "peptide nucleic acids," which comprise naturally-occurring or modified nucleic
25 acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded.

The term "complement of a nucleic acid molecule" refers to a nucleic acid molecule having a complementary nucleotide sequence and reverse orientation as compared to a reference nucleotide sequence. For example, the sequence 5'
30 ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "contig" denotes a nucleic acid molecule that has a contiguous stretch of identical or complementary sequence to another nucleic acid molecule. Contiguous sequences are said to "overlap" a given stretch of a nucleic acid molecule either in their entirety or along a partial stretch of the nucleic acid molecule.

35 The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons as compared to a reference nucleic acid molecule that encodes a polypeptide. Degenerate codons contain different

triplets of nucleotides, but encode the same amino acid residue (*i.e.*, GAU and GAC triplets each encode Asp).

The term "structural gene" refers to a nucleic acid molecule that is transcribed into messenger RNA (mRNA), which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

An "isolated nucleic acid molecule" is a nucleic acid molecule that is not integrated in the genomic DNA of an organism. For example, a DNA molecule that encodes a growth factor that has been separated from the genomic DNA of a cell is an isolated DNA molecule. Another example of an isolated nucleic acid molecule is a chemically-synthesized nucleic acid molecule that is not integrated in the genome of an organism. A nucleic acid molecule that has been isolated from a particular species is smaller than the complete DNA molecule of a chromosome from that species.

A "nucleic acid molecule construct" is a nucleic acid molecule, either single- or double-stranded, that has been modified through human intervention to contain segments of nucleic acid combined and juxtaposed in an arrangement not existing in nature.

"Linear DNA" denotes non-circular DNA molecules having free 5' and 3' ends. Linear DNA can be prepared from closed circular DNA molecules, such as plasmids, by enzymatic digestion or physical disruption.

"Complementary DNA (cDNA)" is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complementary DNA strand. The term "cDNA" also refers to a clone of a cDNA molecule synthesized from an RNA template.

A "promoter" is a nucleotide sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5' non-coding region of a gene, proximal to the transcriptional start site of a structural gene. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. These promoter elements include RNA polymerase binding sites, TATA sequences, CAAT sequences, differentiation-specific elements (DSEs; McGehee *et al.*, *Mol. Endocrinol.* 7:551 (1993)), cyclic AMP response elements (CREs), serum response elements (SREs; Treisman, *Seminars in Cancer Biol.* 1:47 (1990)), glucocorticoid response elements (GREs), and binding sites for other transcription factors, such as CRE/ATF (O'Reilly *et al.*, *J. Biol. Chem.* 267:19938 (1992)), AP2 (Ye *et al.*, *J. Biol. Chem.* 269:25728 (1994)), SP1, cAMP response

element binding protein (CREB; Loeken, *Gene Expr.* 3:253 (1993)) and octamer factors (see, in general, Watson *et al.*, eds., *Molecular Biology of the Gene*, 4th ed. (The Benjamin/Cummings Publishing Company, Inc. 1987), and Lemaigre and Rousseau, *Biochem. J.* 303:1 (1994)). If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Repressible promoters are also known.

A "core promoter" contains essential nucleotide sequences for promoter function, including the TATA box and start of transcription. By this definition, a core promoter may or may not have detectable activity in the absence of specific sequences that may enhance the activity or confer tissue specific activity.

A "regulatory element" is a nucleotide sequence that modulates the activity of a core promoter. For example, a regulatory element may contain a nucleotide sequence that binds with cellular factors enabling transcription exclusively or preferentially in particular cells, tissues, or organelles. These types of regulatory elements are normally associated with genes that are expressed in a "cell-specific," "tissue-specific," or "organelle-specific" manner. For example, the *Zwnt3* regulatory element preferentially induces gene expression in spleen, thymus, spinal cord, and lymph node tissues, as opposed to placenta, lung, and liver tissues.

An "enhancer" is a type of regulatory element that can increase the efficiency of transcription, regardless of the distance or orientation of the enhancer relative to the start site of transcription.

"Heterologous DNA" refers to a DNA molecule, or a population of DNA molecules, that does not exist naturally within a given host cell. DNA molecules heterologous to a particular host cell may contain DNA derived from the host cell species (*i.e.*, endogenous DNA) so long as that host DNA is combined with non-host DNA (*i.e.*, exogenous DNA). For example, a DNA molecule containing a non-host DNA segment encoding a polypeptide operably linked to a host DNA segment comprising a transcription promoter is considered to be a heterologous DNA molecule. Conversely, a heterologous DNA molecule can comprise an endogenous gene operably linked with an exogenous promoter. As another illustration, a DNA molecule comprising a gene derived from a wild-type cell is considered to be heterologous DNA if that DNA molecule is introduced into a mutant cell that lacks the wild-type gene.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides."

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell.

5 Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

A peptide or polypeptide encoded by a non-host DNA molecule is a "heterologous" peptide or polypeptide.

10 An "integrated genetic element" is a segment of DNA that has been incorporated into a chromosome of a host cell after that element is introduced into the cell through human manipulation. Within the present invention, integrated genetic elements are most commonly derived from linearized plasmids that are introduced into the cells by electroporation or other techniques. Integrated genetic elements are passed

15 from the original host cell to its progeny.

A "cloning vector" is a nucleic acid molecule, such as a plasmid, cosmid, or bacteriophage, that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites that allow insertion of a nucleic acid molecule in a determinable fashion

20 without loss of an essential biological function of the vector, as well as nucleotide sequences encoding a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

An "expression vector" is a nucleic acid molecule encoding a gene that is

25 expressed in a host cell. Typically, an expression vector comprises a transcription promoter, a gene, and a transcription terminator. Gene expression is usually placed under the control of a promoter, and such a gene is said to be "operably linked to" the promoter. Similarly, a regulatory element and a core promoter are operably linked if the regulatory element modulates the activity of the core promoter.

30 A "recombinant host" is a cell that contains a heterologous nucleic acid molecule, such as a cloning vector or expression vector. In the present context, an example of a recombinant host is a cell that produces Zwnt3 from an expression vector. In contrast, Zwnt3 can be produced by a cell that is a "natural source" of Zwnt3, and that lacks an expression vector.

35 "Integrative transformants" are recombinant host cells, in which heterologous DNA has become integrated into the genomic DNA of the cells.

A "fusion protein" is a hybrid protein expressed by a nucleic acid molecule comprising nucleotide sequences of at least two genes. For example, a fusion protein can comprise at least part of a Zwnt3 polypeptide fused with a polypeptide that binds an affinity matrix. Such a fusion protein provides a means to isolate large quantities of Zwnt3 using affinity chromatography.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule termed a "ligand." This interaction mediates the effect of the ligand on the cell. Receptors can be membrane bound, cytosolic or nuclear; monomeric (*e.g.*, thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (*e.g.*, PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor). Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. In certain membrane-bound receptors, the extracellular ligand-binding domain and the intracellular effector domain are located in separate polypeptides that comprise the complete functional receptor.

In general, the binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell, which in turn leads to an alteration in the metabolism of the cell. Metabolic events that are often linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids.

The term "secretory signal sequence" denotes a nucleotide sequence that encodes a peptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

An "isolated polypeptide" is a polypeptide that is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature. Typically, a preparation of isolated polypeptide contains the polypeptide in a highly purified form, *i.e.*, at least about 80% pure, at least about 90% pure, at least about 95% pure, greater than 95% pure, or greater than 99% pure. One way to show that a particular protein preparation contains an isolated polypeptide is by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the protein preparation and Coomassie Brilliant Blue staining of the gel. However, the term "isolated" does not

exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

The term "expression" refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a polypeptide encoded by a splice variant of an mRNA transcribed from a gene.

As used herein, the term "immunomodulator" includes cytokines, stem cell growth factors, lymphotoxins, co-stimulatory molecules, hematopoietic factors, and synthetic analogs of these molecules.

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of less than 10^9 M^{-1} .

An "anti-idiotypic antibody" is an antibody that binds with the variable region domain of an immunoglobulin. In the present context, an anti-idiotypic antibody binds with the variable region of an anti-Zwnt3 antibody, and thus, an anti-idiotypic antibody mimics an epitope of Zwnt3.

An "antibody fragment" is a portion of an antibody such as F(ab')_2 , F(ab)_2 , Fab' , Fab , and the like. Regardless of structure, an antibody fragment binds with the same

antigen that is recognized by the intact antibody. For example, an anti-Zwnt3 monoclonal antibody fragment binds with an epitope of Zwnt3.

The term "antibody fragment" also includes a synthetic or a genetically engineered polypeptide that binds to a specific antigen, such as polypeptides consisting of the light chain variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("scFv proteins"), and minimal recognition units consisting of the amino acid residues that mimic the hypervariable region.

A "chimeric antibody" is a recombinant protein that contains the variable domains and complementary determining regions derived from a rodent antibody, while the remainder of the antibody molecule is derived from a human antibody.

"Humanized antibodies" are recombinant proteins in which murine complementarity determining regions of a monoclonal antibody have been transferred from heavy and light variable chains of the murine immunoglobulin into a human variable domain.

As used herein, a "therapeutic agent" is a molecule or atom, which is conjugated to an antibody moiety to produce a conjugate, which is useful for therapy. Examples of therapeutic agents include drugs, toxins, immunomodulators, chelators, boron compounds, photoactive agents or dyes, and radioisotopes.

A "detectable label" is a molecule or atom, which can be conjugated to an antibody moiety to produce a molecule useful for diagnosis. Examples of detectable labels include chelators, photoactive agents, radioisotopes, fluorescent agents, paramagnetic ions, or other marker moieties.

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson *et al.*, *EMBO J.* 4:1075 (1985); Nilsson *et al.*, *Methods Enzymol.* 198:3 (1991)), glutathione S transferase (Smith and Johnson, *Gene* 67:31 (1988)), Glu-Glu affinity tag (Grussenmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7952 (1985)), substance P, FLAG peptide (Hopp *et al.*, *Biotechnology* 6:1204 (1988)), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford *et al.*, *Protein Expression and Purification* 2:95 (1991). Nucleic acid molecules encoding affinity tags are available from commercial suppliers (*e.g.*, Pharmacia Biotech, Piscataway, NJ).

A “naked antibody” is an entire antibody, as opposed to an antibody fragment, which is not conjugated with a therapeutic agent. Naked antibodies include both polyclonal and monoclonal antibodies, as well as certain recombinant antibodies, such as chimeric and humanized antibodies.

5 As used herein, the term “antibody component” includes both an entire antibody and an antibody fragment.

An “immunoconjugate” is a conjugate of an antibody component with a therapeutic agent or a detectable label.

As used herein, the term “antibody fusion protein” refers to a
10 recombinant molecule that comprises an antibody component and a therapeutic agent. Examples of therapeutic agents suitable for such fusion proteins include immunomodulators (“antibody-immunomodulator fusion protein”) and toxins (“antibody-toxin fusion protein”).

A “target polypeptide” or a “target peptide” is an amino acid sequence
15 that comprises at least one epitope, and that is expressed on a target cell, such as a tumor cell, or a cell that carries an infectious agent antigen. T cells recognize peptide epitopes presented by a major histocompatibility complex molecule to a target polypeptide or target peptide and typically lyse the target cell or recruit other immune cells to the site of the target cell, thereby killing the target cell.

20 An “antigenic peptide” is a peptide, which will bind a major histocompatibility complex molecule to form an MHC-peptide complex, which is recognized by a T cell, thereby inducing a cytotoxic lymphocyte response upon presentation to the T cell. Thus, antigenic peptides are capable of binding to an appropriate major histocompatibility complex molecule and inducing a cytotoxic T
25 cells response, such as cell lysis or specific cytokine release against the target cell, which binds or expresses the antigen. The antigenic peptide can be bound in the context of a class I or class II major histocompatibility complex molecule, on an antigen presenting cell or on a target cell.

In eukaryotes, RNA polymerase II catalyzes the transcription of a
30 structural gene to produce mRNA. A nucleic acid molecule can be designed to contain an RNA polymerase II template in which the RNA transcript has a sequence that is complementary to that of a specific mRNA. The RNA transcript is termed an “anti-sense RNA” and a nucleic acid molecule that encodes the anti-sense RNA is termed an “anti-sense gene.” Anti-sense RNA molecules are capable of binding to mRNA
35 molecules, resulting in an inhibition of mRNA translation.

An “anti-sense oligonucleotide specific for Zwnt3” or an “Zwnt3 anti-sense oligonucleotide” is an oligonucleotide having a sequence (a) capable of forming a

stable triplex with a portion of the *Zwnt3* gene, or (b) capable of forming a stable duplex with a portion of an mRNA transcript of the *Zwnt3* gene.

A “ribozyme” is a nucleic acid molecule that contains a catalytic center. The term includes RNA enzymes, self-splicing RNAs, self-cleaving RNAs, and nucleic acid molecules that perform these catalytic functions. A nucleic acid molecule that
5 encodes a ribozyme is termed a “ribozyme gene.”

An “external guide sequence” is a nucleic acid molecule that directs the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, resulting in the cleavage of the mRNA by RNase P. A nucleic acid molecule that encodes an
10 external guide sequence is termed an “external guide sequence gene.”

The term “variant *Zwnt3* gene” refers to nucleic acid molecules that encode a polypeptide having an amino acid sequence that is a modification of SEQ ID NO:2. Such variants include naturally-occurring polymorphisms of *Zwnt3* genes, as well as synthetic genes that contain conservative amino acid substitutions of the amino acid sequence of SEQ ID NO:2. Additional variant forms of *Zwnt3* genes are nucleic acid molecules that contain insertions or deletions of the nucleotide sequences described herein. A variant *Zwnt3* gene can be identified by determining whether the
15 gene hybridizes with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, or its complement, under stringent conditions. The term “variant *Zwnt3*,”
20 however, does not include murine Wnt-8D, which is identified by GenBank Accession No. Z68889 (SEQ ID NO:4, herein), and does not include human Wnt-8B (GenBank Accession No. X91940; SEQ ID NO:6, herein).

Alternatively, variant *Zwnt3* genes can be identified by sequence comparison. Two amino acid sequences have “100% amino acid sequence identity” if
25 the amino acid residues of the two amino acid sequences are the same when aligned for maximal correspondence. Similarly, two nucleotide sequences have “100% nucleotide sequence identity” if the nucleotide residues of the two nucleotide sequences are the same when aligned for maximal correspondence. Sequence comparisons can be performed using standard software programs such as those included in the
30 LASERGENE bioinformatics computing suite, which is produced by DNASTAR (Madison, Wisconsin). Other methods for comparing two nucleotide or amino acid sequences by determining optimal alignment are well-known to those of skill in the art (see, for example, Peruski and Peruski, *The Internet and the New Biology: Tools for Genomic and Molecular Research* (ASM Press, Inc. 1997), Wu *et al.* (eds.),
35 “Information Superhighway and Computer Databases of Nucleic Acids and Proteins,” in *Methods in Gene Biotechnology*, pages 123-151 (CRC Press, Inc. 1997), and Bishop

(ed.), *Guide to Human Genome Computing*, 2nd Edition (Academic Press, Inc. 1998)). Particular methods for determining sequence identity are described below.

Regardless of the particular method used to identify a variant *Zwnt3* gene or variant *Zwnt3* polypeptide, a variant gene or polypeptide encoded by a variant
5 gene may be characterized by the ability to bind specifically to an anti-*Zwnt3* antibody.

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or
10 may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

15 "Paralogs" are distinct but structurally related proteins made by an organism. Paralogs are believed to arise through gene duplication. For example, α -globin, β -globin, and myoglobin are paralogs of each other.

The present invention includes functional fragments of *Zwnt3* genes. Within the context of this invention, a "functional fragment" of a *Zwnt3* gene refers to a
20 nucleic acid molecule that encodes a portion of a *Zwnt3* polypeptide, which specifically binds with an anti-*Zwnt3* antibody. For example, a functional fragment of a *Zwnt3* gene described herein comprises a portion of the nucleotide sequence of SEQ ID NO:1, and encodes a polypeptide that specifically binds with an anti-*Zwnt3* antibody.

Due to the imprecision of standard analytical methods, molecular
25 weights and lengths of polymers are understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to $\pm 10\%$.

3. *Production of a Human Zwnt3 Gene*

30 Nucleic acid molecules encoding a human *Zwnt3* gene can be obtained by screening a human cDNA or genomic library using polynucleotide probes based upon SEQ ID NO:1. These techniques are standard and well-established.

As an illustration, a nucleic acid molecule that encodes a human *Zwnt3* gene can be isolated from a human cDNA library. In this case, the first step would be to
35 prepare the cDNA library by isolating RNA from brain tissue, using methods well-known to those of skill in the art. In general, RNA isolation techniques must provide a method

for breaking cells, a means of inhibiting RNase-directed degradation of RNA, and a method of separating RNA from DNA, protein, and polysaccharide contaminants. For example, total RNA can be isolated by freezing tissue in liquid nitrogen, grinding the frozen tissue with a mortar and pestle to lyse the cells, extracting the ground tissue with a solution of phenol/chloroform to remove proteins, and separating RNA from the remaining impurities by selective precipitation with lithium chloride (see, for example, Ausubel *et al.* (eds.), *Short Protocols in Molecular Biology*, 3rd Edition, pages 4-1 to 4-6 (John Wiley & Sons 1995) ["Ausubel (1995)"]; Wu *et al.*, *Methods in Gene Biotechnology*, pages 33-41 (CRC Press, Inc. 1997) ["Wu (1997)"]).

Alternatively, total RNA can be isolated from spleen, thymus, or uterine tissue by extracting ground tissue with guanidinium isothiocyanate, extracting with organic solvents, and separating RNA from contaminants using differential centrifugation (see, for example, Chirgwin *et al.*, *Biochemistry* 18:52 (1979); Ausubel (1995) at pages 4-1 to 4-6; Wu (1997) at pages 33-41).

In order to construct a cDNA library, poly(A)⁺ RNA must be isolated from a total RNA preparation. Poly(A)⁺ RNA can be isolated from total RNA using the standard technique of oligo(dT)-cellulose chromatography (see, for example, Aviv and Leder, *Proc. Nat'l Acad. Sci. USA* 69:1408 (1972); Ausubel (1995) at pages 4-11 to 4-12).

Double-stranded cDNA molecules are synthesized from poly(A)⁺ RNA using techniques well-known to those in the art. (see, for example, Wu (1997) at pages 41-46). Moreover, commercially available kits can be used to synthesize double-stranded cDNA molecules. For example, such kits are available from Life Technologies, Inc. (Gaithersburg, MD), CLONTECH Laboratories, Inc. (Palo Alto, CA), Promega Corporation (Madison, WI) and STRATAGENE (La Jolla, CA).

Various cloning vectors are appropriate for the construction of a cDNA library. For example, a cDNA library can be prepared in a vector derived from bacteriophage, such as a λ gt10 vector. See, for example, Huynh *et al.*, "Constructing and Screening cDNA Libraries in λ gt10 and λ gt11," in *DNA Cloning: A Practical Approach Vol. I*, Glover (ed.), page 49 (IRL Press, 1985); Wu (1997) at pages 47-52.

Alternatively, double-stranded cDNA molecules can be inserted into a plasmid vector, such as a PBLUESCRIPT vector (STRATAGENE; La Jolla, CA), a LAMDA GEM-4 (Promega Corp.) or other commercially available vectors. Suitable cloning vectors also can be obtained from the American Type Culture Collection (Manassas, VA).

To amplify the cloned cDNA molecules, the cDNA library is inserted into a prokaryotic host, using standard techniques. For example, a cDNA library can be

introduced into competent *E. coli* DH5 cells, which can be obtained, for example, from Life Technologies, Inc. (Gaithersburg, MD).

A human genomic library can be prepared by means well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327).

5 Genomic DNA can be isolated by lysing tissue with the detergent Sarkosyl, digesting the lysate with proteinase K, clearing insoluble debris from the lysate by centrifugation, precipitating nucleic acid from the lysate using isopropanol, and purifying resuspended DNA on a cesium chloride density gradient.

DNA fragments that are suitable for the production of a genomic library
10 can be obtained by the random shearing of genomic DNA or by the partial digestion of genomic DNA with restriction endonucleases. Genomic DNA fragments can be inserted into a vector, such as a bacteriophage or cosmid vector, in accordance with conventional techniques, such as the use of restriction enzyme digestion to provide appropriate termini, the use of alkaline phosphatase treatment to avoid undesirable joining of DNA molecules,
15 and ligation with appropriate ligases. Techniques for such manipulation are well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327).

Nucleic acid molecules that encode a human *Zwnt3* gene can also be obtained using the polymerase chain reaction (PCR) with oligonucleotide primers
20 having nucleotide sequences that are based upon the nucleotide sequences of the human *Zwnt3* gene, as described herein. General methods for screening libraries with PCR are provided by, for example, Yu *et al.*, "Use of the Polymerase Chain Reaction to Screen Phage Libraries," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 211-215 (Humana Press, Inc. 1993).
25 Moreover, techniques for using PCR to isolate related genes are described by, for example, Preston, "Use of Degenerate Oligonucleotide Primers and the Polymerase Chain Reaction to Clone Gene Family Members," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 317-337 (Humana Press, Inc. 1993).

30 Alternatively, human genomic libraries can be obtained from commercial sources such as Research Genetics (Huntsville, AL) and the American Type Culture Collection (Manassas, VA).

A library containing cDNA or genomic clones can be screened with one or more polynucleotide probes based upon SEQ ID NO:1, using standard methods (see, for
35 example, Ausubel (1995) at pages 6-1 to 6-11).

Anti-Zwnt3 antibodies, produced as described below, can also be used to isolate DNA sequences that encode human *Zwnt3* genes from cDNA libraries. For

example, the antibodies can be used to screen λ gt11 expression libraries, or the antibodies can be used for immunoscreening following hybrid selection and translation (see, for example, Ausubel (1995) at pages 6-12 to 6-16; Margolis *et al.*, "Screening λ expression libraries with antibody and protein probes," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 1-14 (Oxford University Press 1995)).

As an alternative, a *Zwnt3* gene can be obtained by synthesizing nucleic acid molecules using mutually priming long oligonucleotides and the nucleotide sequences described herein (see, for example, Ausubel (1995) at pages 8-8 to 8-9). Established techniques using the polymerase chain reaction provide the ability to synthesize DNA molecules at least two kilobases in length (Adang *et al.*, *Plant Molec. Biol.* 21:1131 (1993), Bambot *et al.*, *PCR Methods and Applications* 2:266 (1993), Dillon *et al.*, "Use of the Polymerase Chain Reaction for the Rapid Construction of Synthetic Genes," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 263-268, (Humana Press, Inc. 1993), and Holowachuk *et al.*, *PCR Methods Appl.* 4:299 (1995)).

The nucleic acid molecules of the present invention can also be synthesized with "gene machines" using protocols such as the phosphoramidite method. If chemically-synthesized double stranded DNA is required for an application such as the synthesis of a gene or a gene fragment, then each complementary strand is made separately. The production of short genes (60 to 80 base pairs) is technically straightforward and can be accomplished by synthesizing the complementary strands and then annealing them. For the production of longer genes (>300 base pairs), however, special strategies may be required, because the coupling efficiency of each cycle during chemical DNA synthesis is seldom 100%. To overcome this problem, synthetic genes (double-stranded) are assembled in modular form from single-stranded fragments that are from 20 to 100 nucleotides in length. For reviews on polynucleotide synthesis, see, for example, Glick and Pasternak, *Molecular Biotechnology, Principles and Applications of Recombinant DNA* (ASM Press 1994), Itakura *et al.*, *Annu. Rev. Biochem.* 53:323 (1984), and Climie *et al.*, *Proc. Nat'l Acad. Sci. USA* 87:633 (1990).

The sequence of a *Zwnt3* cDNA or *Zwnt3* genomic fragment can be determined using standard methods. *Zwnt3* polynucleotide sequences disclosed herein can also be used as probes or primers to clone 5' non-coding regions of a *Zwnt3* gene. Promoter elements from a *Zwnt3* gene can be used to direct the expression of heterologous genes in, for example, brain tissue of transgenic animals or patients undergoing gene therapy. The identification of genomic fragments containing a *Zwnt3* promoter or regulatory element can be achieved using well-established techniques, such as deletion analysis (see, generally, Ausubel (1995)).

Cloning of 5' flanking sequences also facilitates production of *Zwnt3* proteins by "gene activation," as disclosed in U.S. Patent No. 5,641,670. Briefly, expression of an endogenous *Zwnt3* gene in a cell is altered by introducing into the *Zwnt3* locus a DNA construct comprising at least a targeting sequence, a regulatory sequence, an exon, and an unpaired splice donor site. The targeting sequence is a *Zwnt3* 5' non-coding sequence that permits homologous recombination of the construct with the endogenous *Zwnt3* locus, whereby the sequences within the construct become operably linked with the endogenous *Zwnt3* coding sequence. In this way, an endogenous *Zwnt3* promoter can be replaced or supplemented with other regulatory sequences to provide enhanced, tissue-specific, or otherwise regulated expression.

4. Production of *Zwnt3* Gene Variants

The present invention provides a variety of nucleic acid molecules, including DNA and RNA molecules, that encode the *Zwnt3* polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. SEQ ID NO:3 is a degenerate nucleotide sequence that encompasses all nucleic acid molecules that encode the *Zwnt3* polypeptide of SEQ ID NO:2. Those skilled in the art will recognize that the degenerate sequence of SEQ ID NO:3 also provides all RNA sequences encoding SEQ ID NO:2, by substituting U for T. Thus, the present invention contemplates *Zwnt3* polypeptide-encoding nucleic acid molecules comprising SEQ ID NO:1, and their RNA equivalents.

Table 1 sets forth the one-letter codes used within SEQ ID NO:3 to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C or T, and its complement R denotes A or G, A being complementary to T, and G being complementary to C.

Table 1

Nucleotide	Resolution	Complement	Resolution
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A G	Y	C T
Y	C T	R	A G
M	A C	K	G T
K	G T	M	A C
S	C G	S	C G
W	A T	W	A T
H	A C T	D	A G T
B	C G T	V	A C G
V	A C G	B	C G T
D	A G T	H	A C T
N	A C G T	N	A C G T

The degenerate codons used in SEQ ID NO:3, encompassing all possible
5 codons for a given amino acid, are set forth in Table 2.

Table 2

Amino Acid	One Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn Asp	B		RAY
Glu Gln	Z		SAR
Any	X		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding an amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO:2. Variant sequences can be readily tested for functionality as described herein.

Different species can exhibit "preferential codon usage." In general, see, Grantham *et al.*, *Nuc. Acids Res.* 8:1893 (1980), Haas *et al.* *Curr. Biol.* 6:315 (1996), Wain-Hobson *et al.*, *Gene* 13:355 (1981), Grosjean and Fiers, *Gene* 18:199 (1982), Holm, *Nuc. Acids Res.* 14:3075 (1986), Ikemura, *J. Mol. Biol.* 158:573 (1982), Sharp and Matassi, *Curr. Opin. Genet. Dev.* 4:851 (1994), Kane, *Curr. Opin. Biotechnol.* 6:494 (1995), and Makrides, *Microbiol. Rev.* 60:512 (1996). As used herein, the term "preferential codon usage" or "preferential codons" is a term of art referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of the possible codons encoding each amino acid (see Table 2). For example, the amino acid threonine (Thr) may be encoded by ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect cells, yeast, viruses or bacteria, different Thr codons may be preferential. Preferential codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species. Therefore, the degenerate codon sequence disclosed in SEQ ID NO:3 serves as a template for optimizing expression of polynucleotides in various cell types and species commonly used in the art and disclosed herein. Sequences containing preferential codons can be tested and optimized for expression in various species, and tested for functionality as disclosed herein.

The present invention further provides variant polypeptides and nucleic acid molecules that represent counterparts from other species (orthologs). These species include, but are not limited to mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are Zwnt3 polypeptides from other mammalian species, including porcine, ovine, bovine, canine, feline, equine, and other primate polypeptides. Orthologs of human Zwnt3 can be

cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses Zwnt3 as disclosed herein. Suitable sources of mRNA can be identified by probing northern
5 blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line.

A Zwnt3-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned using
10 the polymerase chain reaction with primers designed from the representative human Zwnt3 sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to Zwnt3 polypeptide. Similar techniques can also be applied to the isolation of genomic clones.

Those skilled in the art will recognize that the sequence disclosed in
15 SEQ ID NO:1 represents a single allele of human Zwnt3, and that allelic variation and alternative splicing are expected to occur. Allelic variants of this sequence can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the nucleotide sequence shown in SEQ ID
20 NO:1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins, which are allelic variants of SEQ ID NO:2. cDNA molecules generated from alternatively spliced mRNAs, which retain the properties of the Zwnt3 polypeptide are included within the scope of the present invention, as are polypeptides encoded by such
25 cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

Within certain embodiments of the invention, the isolated nucleic acid molecules can hybridize under stringent conditions to nucleic acid molecules
30 comprising nucleotide sequences disclosed herein. For example, such nucleic acid molecules can hybridize under stringent conditions to nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO:1, to nucleic acid molecules consisting of the nucleotide sequence of SEQ ID NO:1, or to nucleic acid molecules consisting of a nucleotide sequence complementary to SEQ ID NO:1. In general,
35 stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the

temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe.

A pair of nucleic acid molecules, such as DNA-DNA, RNA-RNA and DNA-RNA, can hybridize if the nucleotide sequences have some degree of complementarity. Hybrids can tolerate mismatched base pairs in the double helix, but the stability of the hybrid is influenced by the degree of mismatch. The T_m of the mismatched hybrid decreases by 1°C for every 1-1.5% base pair mismatch. Varying the stringency of the hybridization conditions allows control over the degree of mismatch that will be present in the hybrid. The degree of stringency increases as the hybridization temperature increases and the ionic strength of the hybridization buffer decreases. Stringent hybridization conditions encompass temperatures of about 5-25°C below the T_m of the hybrid and a hybridization buffer having up to 1 M Na⁺. Higher degrees of stringency at lower temperatures can be achieved with the addition of formamide, which reduces the T_m of the hybrid about 1°C for each 1% formamide in the buffer solution. Generally, such stringent conditions include temperatures of 20-70°C and a hybridization buffer containing up to 6x SSC and 0-50% formamide. A higher degree of stringency can be achieved at temperatures of from 40-70°C with a hybridization buffer having up to 4x SSC and from 0-50% formamide. Highly stringent conditions typically encompass temperatures of 42-70°C with a hybridization buffer having up to 1x SSC and 0-50% formamide. Different degrees of stringency can be used during hybridization and washing to achieve maximum specific binding to the target sequence. Typically, the washes following hybridization are performed at increasing degrees of stringency to remove non-hybridized polynucleotide probes from hybridized complexes.

The above conditions are meant to serve as a guide and it is well within the abilities of one skilled in the art to adapt these conditions for use with a particular polypeptide hybrid. The T_m for a specific target sequence is the temperature (under defined conditions) at which 50% of the target sequence will hybridize to a perfectly matched probe sequence. Those conditions which influence the T_m include, the size and base pair content of the polynucleotide probe, the ionic strength of the hybridization solution, and the presence of destabilizing agents in the hybridization solution. Numerous equations for calculating T_m are known in the art, and are specific for DNA, RNA and DNA-RNA hybrids and polynucleotide probe sequences of varying length (see, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition (Cold Spring Harbor Press 1989); Ausubel *et al.*, (eds.), *Current Protocols in Molecular Biology* (John Wiley and Sons, Inc. 1987); Berger and Kimmel (eds.), *Guide to Molecular Cloning Techniques*, (Academic Press, Inc. 1987); and Wetmur,

Crit. Rev. Biochem. Mol. Biol. 26:227 (1990)). Sequence analysis software such as OLIGO 6.0 (LSR; Long Lake, MN) and *Primer Premier 4.0* (Premier Biosoft International; Palo Alto, CA), as well as sites on the Internet, are available tools for analyzing a given sequence and calculating T_m based on user defined criteria. Such
5 programs can also analyze a given sequence under defined conditions and identify suitable probe sequences. Typically, hybridization of longer polynucleotide sequences, >50 base pairs, is performed at temperatures of about 20-25°C below the calculated T_m . For smaller probes, <50 base pairs, hybridization is typically carried out at the T_m or 5-10°C below. This allows for the maximum rate of hybridization for DNA-DNA and
10 DNA-RNA hybrids.

The length of the polynucleotide sequence influences the rate and stability of hybrid formation. Smaller probe sequences, <50 base pairs, reach equilibrium with complementary sequences rapidly, but may form less stable hybrids. Incubation times of anywhere from minutes to hours can be used to achieve hybrid
15 formation. Longer probe sequences come to equilibrium more slowly, but form more stable complexes even at lower temperatures. Incubations are allowed to proceed overnight or longer. Generally, incubations are carried out for a period equal to three times the calculated Cot time. Cot time, the time it takes for the polynucleotide sequences to reassociate, can be calculated for a particular sequence by methods known
20 in the art.

The base pair composition of polynucleotide sequence will effect the thermal stability of the hybrid complex, thereby influencing the choice of hybridization temperature and the ionic strength of the hybridization buffer. A-T pairs are less stable than G-C pairs in aqueous solutions containing sodium chloride. Therefore, the higher
25 the G-C content, the more stable the hybrid. Even distribution of G and C residues within the sequence also contribute positively to hybrid stability. In addition, the base pair composition can be manipulated to alter the T_m of a given sequence. For example, 5-methyldeoxycytidine can be substituted for deoxycytidine and 5-bromodeoxuridine can be substituted for thymidine to increase the T_m , whereas 7-deaz-2'-deoxyguanosine
30 can be substituted for guanosine to reduce dependence on T_m .

The ionic concentration of the hybridization buffer also affects the stability of the hybrid. Hybridization buffers generally contain blocking agents such as Denhardt's solution (Sigma Chemical Co., St. Louis, Mo.), denatured salmon sperm DNA, tRNA, milk powders (BLOTTO), heparin or SDS, and a Na^+ source, such as SSC
35 (1x SSC: 0.15 M sodium chloride, 15 mM sodium citrate) or SSPE (1x SSPE: 1.8 M NaCl, 10 mM NaH_2PO_4 , 1 mM EDTA, pH 7.7). By decreasing the ionic concentration of the buffer, the stability of the hybrid is increased. Typically, hybridization buffers

contain from between 10 mM - 1 M Na⁺. The addition of destabilizing or denaturing agents such as formamide, tetraalkylammonium salts, guanidinium cations or thiocyanate cations to the hybridization solution will alter the T_m of a hybrid. Typically, formamide is used at a concentration of up to 50% to allow incubations to be
5 carried out at more convenient and lower temperatures. Formamide also acts to reduce non-specific background when using RNA probes.

As an illustration, a nucleic acid molecule encoding a variant Zwnt3 polypeptide can be hybridized with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) at 42°C overnight in a solution
10 comprising 50% formamide, 5xSSC (1xSSC: 0.15 M sodium chloride and 15 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution (100x Denhardt's solution: 2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone, and 2% (w/v) bovine serum albumin, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA. One of skill in the art can devise variations of these hybridization
15 conditions. For example, the hybridization mixture can be incubated at a higher temperature, such as about 65°C, in a solution that does not contain formamide. Moreover, premixed hybridization solutions are available (*e.g.*, EXPRESSHYB Hybridization Solution from CLONTECH Laboratories, Inc.), and hybridization can be performed according to the manufacturer's instructions.

20 Following hybridization, the nucleic acid molecules can be washed to remove non-hybridized nucleic acid molecules under stringent conditions, or under highly stringent conditions. Typical stringent washing conditions include washing in a solution of 0.5x - 2x SSC with 0.1% sodium dodecyl sulfate (SDS) at 55 - 65°C. For example, certain nucleic acid molecules encoding a variant Zwnt3 polypeptide
25 remained hybridized following stringent washing conditions with a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 (or its complement), in which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C, including 0.5x SSC with 0.1% SDS at 55°C, or 2xSSC with 0.1% SDS at 65°C. One of skill in the art can readily devise equivalent conditions, for example, by substituting the
30 SSPE for SSC in the wash solution.

Typical highly stringent washing conditions include washing in a solution of 0.1x - 0.2x SSC with 0.1% sodium dodecyl sulfate (SDS) at 50 - 65°C. As an illustration, certain nucleic acid molecules encoding a variant Zwnt3 polypeptide remained hybridized following highly stringent washing conditions with a nucleic acid
35 molecule consisting of the nucleotide sequence of SEQ ID NO:1 (or its complement), in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 -

65°C, including 0.1x SSC with 0.1% SDS at 50°C, or 0.2xSSC with 0.1% SDS at 65°C.

The present invention also provides isolated Zwnt3 polypeptides that have a substantially similar sequence identity to the polypeptide of SEQ ID NO:2, or orthologs. The term “substantially similar sequence identity” is used herein to denote polypeptides having 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to the sequence shown in SEQ ID NO:2, with the proviso that such polypeptides include neither murine Wnt-8D (GenBank Accession No. Z68889; SEQ ID NO:4) nor human Wnt-8B (GenBank Accession No. X91940; SEQ ID NO:6).

The present invention also contemplates Zwnt3 variant nucleic acid molecules that can be identified using two criteria: a determination of the similarity between the encoded polypeptide with the amino acid sequence of SEQ ID NO:2, and a hybridization assay, as described above. Such Zwnt3 variants include nucleic acid molecules (1) that remain hybridized following stringent washing conditions with a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 (or its complement), in which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C, and (2) that encode a polypeptide having 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence of SEQ ID NO:2, with the proviso that such polypeptides include neither murine Wnt-8D (GenBank Accession No. Z68889; SEQ ID NO:4) nor human Wnt-8B (GenBank Accession No. X91940; SEQ ID NO:6).

Alternatively, Zwnt3 variants can be characterized as nucleic acid molecules (1) that remain hybridized following highly stringent washing conditions with a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 (or its complement), in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 - 65°C, and (2) that encode a polypeptide having 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence of SEQ ID NO:2, with the proviso that such polypeptides include neither murine Wnt-8D (GenBank Accession No. Z68889; SEQ ID NO:4) nor human Wnt-8B (GenBank Accession No. X91940; SEQ ID NO:6).

Percent sequence identity is determined by conventional methods. See, for example, Altschul *et al.*, *Bull. Math. Bio.* 48:603 (1986), and Henikoff and Henikoff, *Proc. Nat'l Acad. Sci. USA* 89:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the “BLOSUM62” scoring matrix of Henikoff and Henikoff (*ibid.*) as shown in Table 3 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as: ([Total number of identical

matches]/ [length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences])(100).

Table 3

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

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Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative Zwnt3 variant. The FASTA algorithm is described by Pearson and Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444 (1988), and by Pearson, *Meth. Enzymol.* 183:63 (1990). Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (e.g., SEQ ID NO:2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then rescored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, *J. Mol. Biol.* 48:444 (1970); Sellers, *SIAM J. Appl. Math.* 26:787 (1974)), which allows for amino acid insertions and deletions. Illustrative parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol.* 183:63 (1990).

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from three to six, most preferably three, with other parameters set as described above.

The present invention includes nucleic acid molecules that encode a polypeptide having a conservative amino acid change, compared with the amino acid sequence of SEQ ID NO:2. That is, variants can be obtained that contain one or more amino acid substitutions of SEQ ID NO:2, in which an alkyl amino acid is substituted for an alkyl amino acid in a Zwnt3 amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in a Zwnt3 amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in a Zwnt3 amino acid sequence, a hydroxy-containing amino acid is substituted for a hydroxy-

containing amino acid in a *Zwnt3* amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in a *Zwnt3* amino acid sequence, a basic amino acid is substituted for a basic amino acid in a *Zwnt3* amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in a *Zwnt3* amino acid sequence.

Among the common amino acids, for example, a “conservative amino acid substitution” is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) serine and threonine, (4) aspartate and glutamate, (5) glutamine and asparagine, and (6) lysine, arginine and histidine.

The BLOSUM62 table is an amino acid substitution matrix derived from about 2,000 local multiple alignments of protein sequence segments, representing highly conserved regions of more than 500 groups of related proteins (Henikoff and Henikoff, *Proc. Nat'l Acad. Sci. USA* 89:10915 (1992)). Accordingly, the BLOSUM62 substitution frequencies can be used to define conservative amino acid substitutions that may be introduced into the amino acid sequences of the present invention. Although it is possible to design amino acid substitutions based solely upon chemical properties (as discussed above), the language “conservative amino acid substitution” preferably refers to a substitution represented by a BLOSUM62 value of greater than -1. For example, an amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0, 1, 2, or 3. According to this system, preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (e.g., 1, 2 or 3), while more preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 2 (e.g., 2 or 3).

Particular variants of *Zwnt3* are characterized by having greater than 96%, at least 97%, at least 98%, or at least 99% sequence identity to the corresponding amino acid sequence (e.g., SEQ ID NO:2), wherein the variation in amino acid sequence is due to one or more conservative amino acid substitutions.

Conservative amino acid changes in a *Zwnt3* gene can be introduced by substituting nucleotides for the nucleotides recited in SEQ ID NO:1. Such “conservative amino acid” variants can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like (see Ausubel (1995) at pages 8-10 to 8-22; and McPherson (ed.), *Directed Mutagenesis: A Practical Approach* (IRL Press 1991)).

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-

hydroxyproline, N-methylglycine, allo-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipercolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is typically carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson *et al.*, *J. Am. Chem. Soc.* 113:2722 (1991), Ellman *et al.*, *Methods Enzymol.* 202:301 (1991), Chung *et al.*, *Science* 259:806 (1993), and Chung *et al.*, *Proc. Nat'l Acad. Sci. USA* 90:10145 (1993).

In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti *et al.*, *J. Biol. Chem.* 271:19991 (1996)). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (*e.g.*, phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (*e.g.*, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide *et al.*, *Biochem.* 33:7470 (1994). Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, *Protein Sci.* 2:395 (1993)).

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for Zwnt3 amino acid residues.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081 (1989), Bass *et al.*, *Proc. Nat'l Acad. Sci. USA* 88:4498 (1991), Coombs and Corey, "Site-Directed Mutagenesis and Protein Engineering," in *Proteins: Analysis and Design*, Angeletti (ed.), pages 259-311 (Academic Press, Inc. 1998)). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the

resultant mutant molecules are tested for biological activity as disclosed below to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, *J. Biol. Chem.* 271:4699 (1996). The identities of essential amino acids can also be inferred from analysis of homologies with other Wnt proteins.

5 The location of Zwnt3 receptor binding domains can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, *Science* 255:306 (1992), Smith *et al.*, *J. Mol. Biol.* 224:899 (1992), and Wlodaver *et al.*,
10 *FEBS Lett.* 309:59 (1992). Moreover, Zwnt3 labeled with biotin or FITC can be used for expression cloning of Zwnt3 receptors.

 Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (*Science* 241:53 (1988)) or Bowie and Sauer (*Proc. Nat'l Acad. Sci. USA* 86:2152 (1989)). Briefly, these authors disclose methods for simultaneously
15 randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (*e.g.*, Lowman *et al.*, *Biochem.* 30:10832 (1991), Ladner *et al.*,
20 U.S. Patent No. 5,223,409, Huse, international publication No. WO 92/06204, and region-directed mutagenesis (Derbyshire *et al.*, *Gene* 46:145 (1986), and Ner *et al.*, *DNA* 7:127, (1988)).

 Variants of the disclosed Zwnt3 nucleotide and polypeptide sequences can also be generated through DNA shuffling as disclosed by Stemmer, *Nature* 370:389
25 (1994), Stemmer, *Proc. Nat'l Acad. Sci. USA* 91:10747 (1994), and international publication No. WO 97/20078. Briefly, variant DNA molecules are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This
30 technique can be modified by using a family of parent DNA molecules, such as allelic variants or DNAs from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

35 Mutagenesis methods as disclosed herein can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode biologically active

polypeptides, or polypeptides that bind with anti-Zwnt3 antibodies, can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

5 The present invention also includes "functional fragments" of Zwnt3 polypeptides and nucleic acid molecules encoding such functional fragments. Routine deletion analyses of nucleic acid molecules can be performed to obtain functional fragments of a nucleic acid molecule that encodes a Zwnt3 polypeptide. As an illustration, DNA molecules having the nucleotide sequence of SEQ ID NO:1 can be
10 digested with *Bal31* nuclease to obtain a series of nested deletions. The fragments can then be inserted into expression vectors in proper reading frame, and the expressed polypeptides can be isolated and tested for the ability to bind anti-Zwnt3 antibodies. One alternative to exonuclease digestion is to use oligonucleotide-directed mutagenesis to introduce deletions or stop codons to specify production of a desired fragment.
15 Alternatively, particular fragments of a *Zwnt3* gene can be synthesized using the polymerase chain reaction.

 Methods for identifying functional domains are well-known to those of skill in the art. For example, studies on the truncation at either or both termini of interferons have been summarized by Horisberger and Di Marco, *Pharmac. Ther.*
20 66:507 (1995). Moreover, standard techniques for functional analysis of proteins are described by, for example, Treuter *et al.*, *Molec. Gen. Genet.* 240:113 (1993), Content *et al.*, "Expression and preliminary deletion analysis of the 42 kDa 2-5A synthetase induced by human interferon," in *Biological Interferon Systems, Proceedings of ISIR-TNO Meeting on Interferon Systems*, Cantell (ed.), pages 65-72 (Nijhoff 1987),
25 Herschman, "The EGF Receptor," in *Control of Animal Cell Proliferation, Vol. 1*, Boynton *et al.*, (eds.) pages 169-199 (Academic Press 1985), Coumailleau *et al.*, *J. Biol. Chem.* 270:29270 (1995); Fukunaga *et al.*, *J. Biol. Chem.* 270:25291 (1995); Yamaguchi *et al.*, *Biochem. Pharmacol.* 50:1295 (1995), and Meisel *et al.*, *Plant Molec. Biol.* 30:1 (1996).

30 The present invention also contemplates functional fragments of a *Zwnt3* gene that has amino acid changes, compared with the amino acid sequence of SEQ ID NO:2. A variant *Zwnt3* gene can be identified on the basis of structure by determining the level of identity with nucleotide and amino acid sequences of SEQ ID NOs:1 and 2, as discussed above. An alternative approach to identifying a variant gene on the basis
35 of structure is to determine whether a nucleic acid molecule encoding a potential variant *Zwnt3* gene can hybridize to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, as discussed above.

The present invention also provides polypeptide fragments or peptides comprising an epitope-bearing portion of a Zwnt3 polypeptide described herein. Such fragments or peptides may comprise an "immunogenic epitope," which is a part of a protein that elicits an antibody response when the entire protein is used as an immunogen. Immunogenic epitope-bearing peptides can be identified using standard methods (see, for example, Geysen *et al.*, *Proc. Nat'l Acad. Sci. USA* 81:3998 (1983)).

In contrast, polypeptide fragments or peptides may comprise an "antigenic epitope," which is a region of a protein molecule to which an antibody can specifically bind. Certain epitopes consist of a linear or contiguous stretch of amino acids, and the antigenicity of such an epitope is not disrupted by denaturing agents. It is known in the art that relatively short synthetic peptides that can mimic epitopes of a protein can be used to stimulate the production of antibodies against the protein (see, for example, Sutcliffe *et al.*, *Science* 219:660 (1983)). Accordingly, antigenic epitope-bearing peptides and polypeptides of the present invention are useful to raise antibodies that bind with the polypeptides described herein.

Antigenic epitope-bearing peptides and polypeptides can contain at least four to ten amino acids, at least ten to fifteen amino acids, or about 15 to about 30 amino acids of SEQ ID NO:2. Such epitope-bearing peptides and polypeptides can be produced by fragmenting a Zwnt3 polypeptide, or by chemical peptide synthesis, as described herein. Moreover, epitopes can be selected by phage display of random peptide libraries (see, for example, Lane and Stephen, *Curr. Opin. Immunol.* 5:268 (1993), and Cortese *et al.*, *Curr. Opin. Biotechnol.* 7:616 (1996)). Standard methods for identifying epitopes and producing antibodies from small peptides that comprise an epitope are described, for example, by Mole, "Epitope Mapping," in *Methods in Molecular Biology*, Vol. 10, Manson (ed.), pages 105-116 (The Humana Press, Inc. 1992), Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in *Monoclonal Antibodies: Production, Engineering, and Clinical Application*, Ritter and Ladyman (eds.), pages 60-84 (Cambridge University Press 1995), and Coligan *et al.* (eds.), *Current Protocols in Immunology*, pages 9.3.1 - 9.3.5 and pages 9.4.1 - 9.4.11 (John Wiley & Sons 1997).

For any Zwnt3 polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence encoding that variant using the information set forth in Tables 1 and 2 above. Moreover, those of skill in the art can use standard software to devise Zwnt3 variants based upon the nucleotide and amino acid sequences described herein. Accordingly, the present invention includes a computer-readable medium encoded with a data structure that provides at least one of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3.

Suitable forms of computer-readable media include magnetic media and optically-readable media. Examples of magnetic media include a hard or fixed drive, a random access memory (RAM) chip, a floppy disk, digital linear tape (DLT), a disk cache, and a ZIP disk. Optically readable media are exemplified by compact discs (*e.g.*, CD-read only memory (ROM), CD-rewritable (RW), and CD-recordable), and digital versatile/video discs (DVD) (*e.g.*, DVD-ROM, DVD-RAM, and DVD+RW).

5. **Production of Zwnt3 Fusion Proteins**

Fusion proteins of Zwnt3 can be used to express Zwnt3 in a recombinant host, and to isolate expressed Zwnt3. One type of fusion protein comprises a peptide that guides a Zwnt3 polypeptide from a recombinant host cell. To direct a Zwnt3 polypeptide into the secretory pathway of a eukaryotic host cell, a secretory signal sequence (also known as a signal peptide, a leader sequence, prepro sequence or pre sequence) is provided in the Zwnt3 expression vector. While the secretory signal sequence may be derived from Zwnt3, a suitable signal sequence may also be derived from another secreted protein or synthesized *de novo*. The secretory signal sequence is operably linked to a Zwnt3-encoding sequence such that the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the nucleotide sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the nucleotide sequence of interest (see, *e.g.*, Welch *et al.*, U.S. Patent No. 5,037,743; Holland *et al.*, U.S. Patent No. 5,143,830).

While the secretory signal sequence of Zwnt3 or another protein produced by mammalian cells (*e.g.*, tissue-type plasminogen activator signal sequence, as described, for example, in U.S. Patent No. 5,641,655) is useful for expression of Zwnt3 in recombinant mammalian hosts, a yeast signal sequence is preferred for expression in yeast cells. Examples of suitable yeast signal sequences are those derived from yeast mating pheromone α -factor (encoded by the *MF α 1* gene), invertase (encoded by the *SUC2* gene), or acid phosphatase (encoded by the *PHO5* gene). See, for example, Romanos *et al.*, "Expression of Cloned Genes in Yeast," in *DNA Cloning 2: A Practical Approach*, 2nd Edition, Glover and Hames (eds.), pages 123-167 (Oxford University Press 1995).

In bacterial cells, it is often desirable to express a heterologous protein as a fusion protein to decrease toxicity, increase stability, and to enhance recovery of the expressed protein. For example, Zwnt3 can be expressed as a fusion protein

comprising a glutathione S-transferase polypeptide. Glutathione S-transferase fusion proteins are typically soluble, and easily purifiable from *E. coli* lysates on immobilized glutathione columns. In similar approaches, a Zwnt3 fusion protein comprising a maltose binding protein polypeptide can be isolated with an amylose resin column, while a fusion protein comprising the C-terminal end of a truncated Protein A gene can be purified using IgG-Sepharose. Established techniques for expressing a heterologous polypeptide as a fusion protein in a bacterial cell are described, for example, by Williams *et al.*, "Expression of Foreign Proteins in *E. coli* Using Plasmid Vectors and Purification of Specific Polyclonal Antibodies," in *DNA Cloning 2: A Practical Approach*, 2nd Edition, Glover and Hames (Eds.), pages 15-58 (Oxford University Press 1995). In addition, commercially available expression systems are available. For example, the PINPOINT Xa protein purification system (Promega Corporation; Madison, WI) provides a method for isolating a fusion protein comprising a polypeptide that becomes biotinylated during expression with a resin that comprises avidin.

Peptide tags that are useful for isolating heterologous polypeptides expressed by either prokaryotic or eukaryotic cells include polyHistidine tags (which have an affinity for nickel-chelating resin), *c-myc* tags, calmodulin binding protein (isolated with calmodulin affinity chromatography), substance P, the RYIRS tag (which binds with anti-RYIRS antibodies), the Glu-Glu tag, and the FLAG tag (which binds with anti-FLAG antibodies). See, for example, Luo *et al.*, *Arch. Biochem. Biophys.* 329:215 (1996), Morganti *et al.*, *Biotechnol. Appl. Biochem.* 23:67 (1996), and Zheng *et al.*, *Gene* 186:55 (1997). Nucleic acid molecules encoding such peptide tags are available, for example, from Sigma-Aldrich Corporation (St. Louis, MO).

Another form of fusion protein comprises a Zwnt3 polypeptide and an immunoglobulin heavy chain constant region, typically an F_C fragment, which contains two constant region domains and a hinge region but lacks the variable region. As an illustration, Chang *et al.*, U.S. Patent No. 5,723,125, describe a fusion protein comprising a human interferon and a human immunoglobulin Fc fragment, in which the C-terminal of the interferon is linked to the N-terminal of the Fc fragment by a peptide linker moiety. An example of a peptide linker is a peptide comprising primarily a T cell inert sequence, which is immunologically inert. An exemplary peptide linker has the amino acid sequence: GGSGG SGGGG SGGGG S (SEQ ID NO:5). In such a fusion protein, an illustrative Fc moiety is a human γ 4 chain, which is stable in solution and has little or no complement activating activity. Accordingly, the present invention contemplates a Zwnt3 fusion protein that comprises a Zwnt3 moiety and a human Fc fragment, wherein the C-terminus of the Zwnt3 moiety is attached to the N-terminus of the Fc fragment via a peptide linker, such as a peptide consisting of the amino acid

sequence of SEQ ID NO:5. The Zwnt3 moiety can be a Zwnt3 molecule or a fragment thereof.

In another variation, a Zwnt3 fusion protein comprises an IgG sequence, a Zwnt3 moiety covalently joined to the aminoterminal end of the IgG sequence, and a signal peptide that is covalently joined to the aminoterminal of the Zwnt3 moiety, wherein the IgG sequence consists of the following elements in the following order: a hinge region, a CH₂ domain, and a CH₃ domain. Accordingly, the IgG sequence lacks a CH₁ domain. The Zwnt3 moiety displays a Zwnt3 activity, as described herein, such as the ability to bind with a Zwnt3 antibody. This general approach to producing fusion proteins that comprise both antibody and nonantibody portions has been described by LaRochelle *et al.*, EP 742830 (WO 95/21258).

Fusion proteins comprising a Zwnt3 moiety and an Fc moiety can be used, for example, as an *in vitro* assay tool. For example, the presence of a Zwnt3 receptor in a biological sample can be detected using a Zwnt3-antibody fusion protein, in which the Zwnt3 moiety is used to target the cognate receptor, and a macromolecule, such as Protein A or anti-Fc antibody, is used to detect the bound fusion protein-receptor complex. Furthermore, such fusion proteins can be used to identify agonists and antagonists that interfere with the binding of Zwnt3 to its receptor.

Moreover, using methods described in the art, hybrid Zwnt3 proteins can be constructed using regions or domains of the inventive Zwnt3 in combination with those of other Wnt family proteins (*e.g.*, human Wnt-1, Wnt-2, Wnt-2B/13, Wnt-3, Wnt-4, Wnt-5A, Wnt-7A, Wnt-8A, Wnt-8B, Wnt-8D, Wnt-10B, Wnt-11, Wnt-14, and Wnt-15), or heterologous proteins (see, for example, Picard, *Cur. Opin. Biology* 5:511 (1994)). These methods allow the determination of the biological importance of larger domains or regions in a polypeptide of interest. Such hybrids may alter reaction kinetics, binding, constrict or expand the substrate specificity, or alter tissue and cellular localization of a polypeptide, and can be applied to polypeptides of unknown structure. For example Horisberger and DiMarco, *Pharmac. Ther.* 66:507 (1995), describe the construction of fusion protein hybrids comprising different interferon- α subtypes, as well as hybrids comprising interferon- α domains from different species.

Fusion proteins can be prepared by methods known to those skilled in the art by preparing each component of the fusion protein and chemically conjugating the components. Alternatively, a polynucleotide encoding both components of the fusion protein in the proper reading frame can be generated using known techniques and expressed by the methods described herein. General methods for enzymatic and chemical cleavage of fusion proteins are described, for example, by Ausubel (1995) at pages 16-19 to 16-25.

6. *Production of Zwnt3 Polypeptides in Cultured Cells*

The polypeptides of the present invention, including full-length polypeptides, functional fragments, and fusion proteins, can be produced in recombinant host cells following conventional techniques. To express a *Zwnt3* gene, a nucleic acid molecule encoding the polypeptide must be operably linked to regulatory sequences that control transcriptional expression in an expression vector and then, introduced into a host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors can include translational regulatory sequences and a marker gene, which is suitable for selection of cells that carry the expression vector.

Expression vectors that are suitable for production of a foreign protein in eukaryotic cells typically contain (1) prokaryotic DNA elements coding for a bacterial replication origin and an antibiotic resistance marker to provide for the growth and selection of the expression vector in a bacterial host; (2) eukaryotic DNA elements that control initiation of transcription, such as a promoter; and (3) DNA elements that control the processing of transcripts, such as a transcription termination/polyadenylation sequence. As discussed above, expression vectors can also include nucleotide sequences encoding a secretory sequence that directs the heterologous polypeptide into the secretory pathway of a host cell. For example, a *Zwnt3* expression vector may comprise a *Zwnt3* gene and a secretory sequence derived from a *Zwnt3* gene or another secreted gene.

Zwnt3 proteins of the present invention may be expressed in mammalian cells. Examples of suitable mammalian host cells include African green monkey kidney cells (Vero; ATCC CRL 1587), human embryonic kidney cells (293-HEK; ATCC CRL 1573), baby hamster kidney cells (BHK-21, BHK-570; ATCC CRL 8544, ATCC CRL 10314), canine kidney cells (MDCK; ATCC CCL 34), Chinese hamster ovary cells (CHO-K1; ATCC CCL61; CHO DG44 (Chasin *et al.*, *Som. Cell. Molec. Genet.* 12:555, 1986)), rat pituitary cells (GH1; ATCC CCL82), HeLa S3 cells (ATCC CCL2.2), rat hepatoma cells (H-4-II-E; ATCC CRL 1548) SV40-transformed monkey kidney cells (COS-1; ATCC CRL 1650) and murine embryonic cells (NIH-3T3; ATCC CRL 1658).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, in which the regulatory signals are associated with a particular gene, which has a high level of expression. Suitable transcriptional and translational

regulatory sequences also can be obtained from mammalian genes, such as actin, collagen, myosin, and metallothionein genes.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse *metallothionein I* gene (Hamer *et al.*, *J. Molec. Appl. Genet.* 1:273 (1982)), the *TK* promoter of *Herpes* virus (McKnight, *Cell* 31:355 (1982)), the *SV40* early promoter (Benoist *et al.*, *Nature* 290:304 (1981)), the *Rous* sarcoma virus promoter (Gorman *et al.*, *Proc. Nat'l Acad. Sci. USA* 79:6777 (1982)), the cytomegalovirus promoter (Foecking *et al.*, *Gene* 45:101 (1980)), and the mouse mammary tumor virus promoter (see, generally, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 163-181 (John Wiley & Sons, Inc. 1996)).

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control *Zwnt3* gene expression in mammalian cells if the prokaryotic promoter is regulated by a eukaryotic promoter (Zhou *et al.*, *Mol. Cell. Biol.* 10:4529 (1990), and Kaufman *et al.*, *Nucl. Acids Res.* 19:4485 (1991)).

An expression vector can be introduced into host cells using a variety of standard techniques including calcium phosphate transfection, liposome-mediated transfection, microprojectile-mediated delivery, electroporation, and the like. The transfected cells can be selected and propagated to provide recombinant host cells that comprise the expression vector stably integrated in the host cell genome. Techniques for introducing vectors into eukaryotic cells and techniques for selecting such stable transformants using a dominant selectable marker are described, for example, by Ausubel (1995) and by Murray (ed.), *Gene Transfer and Expression Protocols* (Humana Press 1991).

For example, one suitable selectable marker is a gene that provides resistance to the antibiotic neomycin. In this case, selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. An exemplary amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (*e.g.*, hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternatively, markers that introduce an altered phenotype, such as green

fluorescent protein, or cell surface proteins (e.g., CD4, CD8, Class I MHC, and placental alkaline phosphatase) may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

5 Zwnt3 polypeptides can also be produced by cultured cells using a viral delivery system. Exemplary viruses for this purpose include adenovirus, herpesvirus, vaccinia virus and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid (for a review, see Becker *et al.*, *Meth. Cell Biol.* 43:161 (1994), and Douglas and Curiel, *Science & Medicine* 4:44 (1997)). Advantages of the adenovirus system include the accommodation of relatively large DNA inserts, the ability to grow to high-titer, the ability to infect a broad range of mammalian cell types, and flexibility that allows use with a large number of available vectors containing different promoters.

10 By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. An option is to delete the essential *E1* gene from the viral vector, which results in the inability to replicate unless the *E1* gene is provided by the host cell. For example, adenovirus vector infected human 293 cells (ATCC Nos. CRL-1573, 45504, 45505) can be grown as adherent cells or in suspension culture at relatively high cell density to produce significant amounts of protein (see Garnier *et al.*, *Cytotechnol.* 15:145 (1994)).

20 Zwnt3 genes may also be expressed in other higher eukaryotic cells, such as avian, fungal, insect, yeast, or plant cells. The baculovirus system provides an efficient means to introduce cloned Zwnt3 genes into insect cells. Suitable expression vectors are based upon the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), and contain well-known promoters such as *Drosophila* heat shock protein (hsp) 70 promoter, *Autographa californica* nuclear polyhedrosis virus immediate-early gene promoter (*ie-1*) and the delayed early 39K promoter, baculovirus p10 promoter, and the *Drosophila* metallothionein promoter. A second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow (Luckow, *et al.*, *J. Virol.* 67:4566 (1993)). This system, which utilizes transfer vectors, is sold in the BAC-to-BAC kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, PFASTBAC (Life Technologies) containing a Tn7 transposon to move the DNA encoding the Zwnt3 polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." See, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971 (1990), Bonning, *et al.*, *J. Gen. Virol.* 75:1551 (1994), and Chazenbalk, and Rapoport, *J. Biol. Chem.* 270:1543 (1995). In addition, transfer vectors can include an in-frame

fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed Zwnt3 polypeptide, for example, a Glu-Glu epitope tag (Grussenmeyer *et al.*, *Proc. Nat'l Acad. Sci.* 82:7952 (1985)). Using a technique known in the art, a transfer vector containing a *Zwnt3* gene is transformed into *E. coli*, and screened for bacmids, which
5 contain an interrupted *lacZ* gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is then isolated using common techniques.

The illustrative PFASTBAC vector can be modified to a considerable degree. For example, the polyhedrin promoter can be removed and substituted with the
10 baculovirus basic protein promoter (also known as *Pcor*, p6.9 or MP promoter), which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins (see, for example, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971 (1990), Bonning, *et al.*, *J. Gen. Virol.* 75:1551 (1994), and Chazenbalk and Rapoport, *J. Biol. Chem.* 270:1543 (1995). In such transfer vector
15 constructs, a short or long version of the basic protein promoter can be used. Moreover, transfer vectors can be constructed which replace the native *Zwnt3* secretory signal sequences with secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honey bee Melittin (Invitrogen Corporation; Carlsbad, CA), or baculovirus gp67 (PharMingen:
20 San Diego, CA) can be used in constructs to replace the native *Zwnt3* secretory signal sequence.

The recombinant virus or bacmid is used to transfect host cells. Suitable insect host cells include cell lines derived from IPLB-Sf-21, a *Spodoptera frugiperda* pupal ovarian cell line, such as Sf9 (ATCC CRL 1711), Sf21AE, and Sf21 (Invitrogen Corporation; San Diego, CA), as well as *Drosophila* Schneider-2 cells, and the HIGH
25 FIVEO cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent No. 5,300,435). Commercially available serum-free media can be used to grow and to maintain the cells. Suitable media are Sf900 II™ (Life Technologies) or ESF 921™ (Expression Systems) for the Sf9 cells; and Ex-cello405™ (JRH Biosciences, Lenexa, KS) or Express FiveO™ (Life Technologies) for the *T. ni* cells. When recombinant virus is
30 used, the cells are typically grown up from an inoculation density of approximately $2-5 \times 10^5$ cells to a density of $1-2 \times 10^6$ cells at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3.

Established techniques for producing recombinant proteins in
35 baculovirus systems are provided by Bailey *et al.*, "Manipulation of Baculovirus Vectors," in *Methods in Molecular Biology, Volume 7: Gene Transfer and Expression Protocols*, Murray (ed.), pages 147-168 (The Humana Press, Inc. 1991), by Patel *et al.*,

“The baculovirus expression system,” in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 205-244 (Oxford University Press 1995), by Ausubel (1995) at pages 16-37 to 16-57, by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995), and by Lucknow, “Insect Cell Expression Technology,”
5 in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 183-218 (John Wiley & Sons, Inc. 1996).

Fungal cells, including yeast cells, can also be used to express the genes described herein. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Suitable promoters
10 for expression in yeast include promoters from *GALI* (galactose), *PGK* (phosphoglycerate kinase), *ADH* (alcohol dehydrogenase), *AOX1* (alcohol oxidase), *HIS4* (histidinol dehydrogenase), and the like. Many yeast cloning vectors have been designed and are readily available. These vectors include YIp-based vectors, such as YIp5, YRp vectors, such as YRp17, YEp vectors such as YEp13 and YCp vectors, such
15 as YCp19. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311, Kawasaki *et al.*, U.S. Patent No. 4,931,373, Brake, U.S. Patent No. 4,870,008, Welch *et al.*, U.S. Patent No. 5,037,743, and Murray *et al.*, U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype
20 determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (*e.g.*, leucine). An illustrative vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki *et al.* (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Additional suitable promoters and terminators for use in
25 yeast include those from glycolytic enzyme genes (see, *e.g.*, Kawasaki, U.S. Patent No. 4,599,311, Kingsman *et al.*, U.S. Patent No. 4,615,974, and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446, 5,063,154, 5,139,936, and 4,661,454.

Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guilliermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson *et al.*, *J. Gen. Microbiol.* 132:3459 (1986), and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells
30 may be utilized according to the methods of McKnight *et al.*, U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino *et al.*, U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

For example, the use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed by Raymond, U.S. Patent No. 5,716,808, Raymond, U.S. Patent No. 5,736,383, Raymond *et al.*, *Yeast* 14:11-23 (1998), and in international publication Nos. WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565.

5 DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which can be linearized prior to transformation. For polypeptide production in *P. methanolica*, a suitable promoter and terminator in the plasmid are provided by a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful promoters include those of the

10 dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, the entire expression segment of the plasmid can be flanked at both ends by host DNA sequences. An illustrative selectable marker for use in *Pichia methanolica* is a *P. methanolica* *ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase

15 (AIRC; EC 4.1.1.21), and which allows *ade2* host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is possible to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are suitable. Electroporation is used to

20 facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. *P. methanolica* cells can be transformed by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (*t*) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

25 Expression vectors can also be introduced into plant protoplasts, intact plant tissues, or isolated plant cells. Methods for introducing expression vectors into plant tissue include the direct infection or co-cultivation of plant tissue with *Agrobacterium tumefaciens*, microprojectile-mediated delivery, DNA injection, electroporation, and the like. See, for example, Horsch *et al.*, *Science* 227:1229 (1985),

30 Klein *et al.*, *Biotechnology* 10:268 (1992), and Miki *et al.*, "Procedures for Introducing Foreign DNA into Plants," in *Methods in Plant Molecular Biology and Biotechnology*, Glick *et al.* (eds.), pages 67-88 (CRC Press, 1993).

Alternatively, *Zwnt3* genes can be expressed in prokaryotic host cells. Suitable promoters that can be used to express *Zwnt3* polypeptides in a prokaryotic host

35 are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the *P_R* and *P_L* promoters of bacteriophage lambda, the *trp*, *recA*, heat shock, *lacUV5*, *tac*, *lpp-lacSpr*, *phoA*, and *lacZ* promoters of *E. coli*,

promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the *int* promoter of bacteriophage lambda, the *bla* promoter of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters have been reviewed by Glick, *J. Ind. Microbiol.* 1:277 (1987), Watson *et al.*,
5 *Molecular Biology of the Gene*, 4th Ed. (Benjamin Cummins 1987), and by Ausubel *et al.* (1995).

Useful prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Suitable strains of *E. coli* include BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE, DH1, DH4I, DH5, DH5I, DH5IF', DH5IMCR, DH10B, DH10B/p3, DH11S, C600, HB101, JM101, JM105, JM109, JM110, K38, RR1, Y1088, Y1089, CSH18, ER1451, and
10 ER1647 (see, for example, Brown (ed.), *Molecular Biology Labfax* (Academic Press 1991)). Suitable strains of *Bacillus subtilis* include BR151, YB886, MI119, MI120, and B170 (see, for example, Hardy, "Bacillus Cloning Methods," in *DNA Cloning: A Practical Approach*, Glover (ed.) (IRL Press 1985)).

When expressing a Zwnt3 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and
20 dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space
25 and recovering the protein, thereby obviating the need for denaturation and refolding.

Methods for expressing proteins in prokaryotic hosts are well-known to those of skill in the art (see, for example, Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems*, 2nd Edition, Glover *et al.* (eds.),
30 page 15 (Oxford University Press 1995), Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, page 137 (Wiley-Liss, Inc. 1995), and Georgiou, "Expression of Proteins in Bacteria," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), page 101 (John Wiley & Sons, Inc. 1996)).

Standard methods for introducing expression vectors into bacterial, yeast, insect, and plant cells are provided, for example, by Ausubel (1995).

General methods for expressing and recovering foreign protein produced by a mammalian cell system are provided by, for example, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 163 (Wiley-Liss, Inc. 1996). Standard techniques for recovering protein produced by a bacterial system is provided by, for example, Grishammer *et al.*, "Purification of over-produced proteins from *E. coli* cells," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 59-92 (Oxford University Press 1995). Established methods for isolating recombinant proteins from a baculovirus system are described by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995).

As an alternative, polypeptides of the present invention can be synthesized by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. These synthesis methods are well-known to those of skill in the art (see, for example, Merrifield, *J. Am. Chem. Soc.* 85:2149 (1963), Stewart *et al.*, "Solid Phase Peptide Synthesis" (2nd Edition), (Pierce Chemical Co. 1984), Bayer and Rapp, *Chem. Pept. Prot.* 3:3 (1986), Atherton *et al.*, *Solid Phase Peptide Synthesis: A Practical Approach* (IRL Press 1989), Fields and Colowick, "Solid-Phase Peptide Synthesis," *Methods in Enzymology Volume 289* (Academic Press 1997), and Lloyd-Williams *et al.*, *Chemical Approaches to the Synthesis of Peptides and Proteins* (CRC Press, Inc. 1997)). Variations in total chemical synthesis strategies, such as "native chemical ligation" and "expressed protein ligation" are also standard (see, for example, Dawson *et al.*, *Science* 266:776 (1994), Hackeng *et al.*, *Proc. Nat'l Acad. Sci. USA* 94:7845 (1997), Dawson, *Methods Enzymol.* 287: 34 (1997), Muir *et al.*, *Proc. Nat'l Acad. Sci. USA* 95:6705 (1998), and Severinov and Muir, *J. Biol. Chem.* 273:16205 (1998)).

7. Isolation of Zwnt3 Polypeptides

The polypeptides of the present invention can be purified to at least about 80% purity, to at least about 90% purity, to at least about 95% purity, or greater than 95% purity with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. The polypeptides of the present invention may also be purified to a pharmaceutically pure state, which is greater than 99.9% pure. Certain purified polypeptide preparations are substantially free of other polypeptides, particularly other polypeptides of animal origin.

Fractionation and/or conventional purification methods can be used to obtain preparations of Zwnt3 purified from natural sources (*e.g.*, brain tissue), and

recombinant Zwnt3 polypeptides and fusion Zwnt3 polypeptides purified from recombinant host cells. In general, ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable chromatographic media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties.

Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Selection of a particular method for polypeptide isolation and purification is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, *Affinity Chromatography: Principles & Methods* (Pharmacia LKB Biotechnology 1988), and Doonan, *Protein Purification Protocols* (The Humana Press 1996).

Additional variations in Zwnt3 isolation and purification can be devised by those of skill in the art. For example, anti-Zwnt3 antibodies, obtained as described below, can be used to isolate large quantities of protein by immunoaffinity purification. Moreover, methods for binding ligands, such as Zwnt3, to receptor polypeptides bound to support media are well known in the art.

The polypeptides of the present invention can also be isolated by exploitation of particular properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins, including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate (Sulkowski, *Trends in Biochem.* 3:1 (1985)). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating

agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (M. Deutscher, (ed.), *Meth. Enzymol.* 182:529 (1990)). Within additional embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (*e.g.*, maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

Zwnt3 polypeptides or fragments thereof may also be prepared through chemical synthesis, as described above. Zwnt3 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; PEGylated or non-PEGylated; and may or may not include an initial methionine amino acid residue.

The present invention also contemplates chemically modified Zwnt3 compositions, in which a Zwnt3 polypeptide is linked with a polymer. Typically, the polymer is water soluble so that the Zwnt3 conjugate does not precipitate in an aqueous environment, such as a physiological environment. An example of a suitable polymer is one that has been modified to have a single reactive group, such as an active ester for acylation, or an aldehyde for alkylation. In this way, the degree of polymerization can be controlled. An example of a reactive aldehyde is polyethylene glycol propionaldehyde, or mono-(C1-C10) alkoxy, or aryloxy derivatives thereof (see, for example, Harris, *et al.*, U.S. Patent No. 5,252,714). The polymer may be branched or unbranched. Moreover, a mixture of polymers can be used to produce Zwnt3 conjugates.

Zwnt3 conjugates used for therapy can comprise pharmaceutically acceptable water-soluble polymer moieties. Suitable water-soluble polymers include polyethylene glycol (PEG), monomethoxy-PEG, mono-(C1-C10)alkoxy-PEG, aryloxy-PEG, poly-(N-vinyl pyrrolidone)PEG, tresyl monomethoxy PEG, PEG propionaldehyde, *bis*-succinimidyl carbonate PEG, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (*e.g.*, glycerol), polyvinyl alcohol, dextran, cellulose, or other carbohydrate-based polymers. Suitable PEG may have a molecular weight from about 600 to about 60,000, including, for example, 5,000, 12,000, 20,000 and 25,000. A Zwnt3 conjugate can also comprise a mixture of such water-soluble polymers. Anti-Zwnt3 antibodies or anti-idiotypic antibodies can also be conjugated with a water-soluble polymer.

The present invention contemplates compositions comprising a peptide or polypeptide described herein. Such compositions can further comprise a carrier. The carrier can be a conventional organic or inorganic carrier. Examples of carriers include water, buffer solution, alcohol, propylene glycol, macrogol, sesame oil, corn oil, and the like.

Peptides and polypeptides of the present invention comprise at least six, at least nine, or at least 15 contiguous amino acid residues of SEQ ID NO:2. Within certain embodiments of the invention, the polypeptides comprise 20, 30, 40, 50, 100, or more contiguous residues of these amino acid sequences. For example, peptides and polypeptides can comprise the following regions of SEQ ID NO:2: amino acid residues 30 to 42, amino acid residues 44 to 62, amino acid residues 69 to 97, amino acid residues 101 to 111, amino acid residues 120 to 138, amino acid residues 143 to 177, amino acid residues 184 to 215, amino acid residues 217 to 231, amino acid residues 217 to 248, amino acid residues 231 to 248, amino acid residues 276 to 297, amino acid residues 321 to 345, amino acid residues 345 to 415, and amino acid residues 321 to 415. Additional polypeptides can comprise at least 15, at least 30, at least 40, or at least 50 contiguous amino acids of such regions of SEQ ID NO:2. Nucleic acid molecules encoding such peptides and polypeptides are useful as polymerase chain reaction primers and probes.

In addition to the uses described above, polynucleotides and polypeptides of the present invention are useful as educational tools in laboratory practicum kits for courses related to genetics and molecular biology, protein chemistry, and antibody production and analysis. Due to its unique polynucleotide and polypeptide sequences, molecules of *Zwnt3* can be used as standards or as "unknowns" for testing purposes. For example, *Zwnt3* polynucleotides can be used as an aid, such as, for example, to teach a student how to prepare expression constructs for bacterial, viral, or mammalian expression, including fusion constructs, wherein *Zwnt3* is the gene to be expressed; for determining the restriction endonuclease cleavage sites of the polynucleotides; determining mRNA and DNA localization of *Zwnt3* polynucleotides in tissues (*i.e.*, by northern and Southern blotting as well as polymerase chain reaction); and for identifying related polynucleotides and polypeptides by nucleic acid hybridization. As an illustration, students will find that *Xho*II digestion of a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 provides fragments of about 42 base pairs, 381 base pairs, and 822 base pairs, and that *Hpa*II digestion yields fragments of about 103 base pairs, 501 base pairs, and 641 base pairs.

Zwnt3 polypeptides can be used as an aid to teach preparation of antibodies; identifying proteins by western blotting; protein purification; determining the weight of expressed *Zwnt3* polypeptides as a ratio to total protein expressed; identifying peptide cleavage sites; coupling amino and carboxyl terminal tags; amino acid sequence analysis, as well as, but not limited to monitoring biological activities of both the native and tagged protein (*i.e.*, protease inhibition) *in vitro* and *in vivo*. For example, students will find that digestion of unglycosylated *Zwnt3* with hydroxylamine

yields six fragments having approximate molecular weights of 319, 7199, 5522, 4566, 21751, and 7165, whereas digestion of unglycosylated Zwnt3 with cyanogen bromide yields fragments having approximate molecular weights of 148, 580, 9945, 1071, 5765, 1793, 3216, 1885, and 22170.

5 Zwnt3 polypeptides can also be used to teach analytical skills such as mass spectrometry, circular dichroism, to determine conformation, especially of the four alpha helices, x-ray crystallography to determine the three-dimensional structure in atomic detail, nuclear magnetic resonance spectroscopy to reveal the structure of proteins in solution. For example, a kit containing the Zwnt3 can be given to the student
10 to analyze. Since the amino acid sequence would be known by the instructor, the protein can be given to the student as a test to determine the skills or develop the skills of the student, the instructor would then know whether or not the student has correctly analyzed the polypeptide. Since every polypeptide is unique, the educational utility of Zwnt3 would be unique unto itself.

15 The antibodies which bind specifically to Zwnt3 can be used as a teaching aid to instruct students how to prepare affinity chromatography columns to purify Zwnt3, cloning and sequencing the polynucleotide that encodes an antibody and thus as a practicum for teaching a student how to design humanized antibodies. The *Zwnt3* gene, polypeptide, or antibody would then be packaged by reagent companies
20 and sold to educational institutions so that the students gain skill in art of molecular biology. Because each gene and protein is unique, each gene and protein creates unique challenges and learning experiences for students in a lab practicum. Such educational kits containing the *Zwnt3* gene, polypeptide, or antibody are considered within the scope of the present invention.

25

8. *Production of Antibodies to Zwnt3 Proteins*

Antibodies to Zwnt3 can be obtained, for example, using as an antigen the product of a Zwnt3 expression vector or Zwnt3 isolated from a natural source. Particularly useful anti-Zwnt3 antibodies "bind specifically" with Zwnt3. Antibodies
30 are considered to be specifically binding if the antibodies exhibit at least one of the following two properties: (1) antibodies bind to Zwnt3 with a threshold level of binding activity, and (2) antibodies do not significantly cross-react with polypeptides related to Zwnt3.

With regard to the first characteristic, antibodies specifically bind if they
35 bind to a Zwnt3 polypeptide, peptide or epitope with a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most

preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, *Ann. NY Acad. Sci.* 51:660 (1949)). With regard to the second characteristic, antibodies do not significantly cross-react with related polypeptide molecules, for example, if they detect Zwnt3, but not known related polypeptides using a standard Western blot analysis. Examples of known related polypeptides are orthologs and proteins from the same species that are members of a protein family. For example, specifically-binding anti-Zwnt3 antibodies bind with Zwnt3, but not with polypeptides such as human Wnt-1, Wnt-2, Wnt-2B/13, Wnt-3, Wnt-4, Wnt-5A, Wnt-7A, Wnt-8A, Wnt-8B, Wnt-10B, Wnt-11, Wnt-14, Wnt-15, and murine Wnt-8D.

Suitable antibodies include antibodies that bind with Zwnt3 in regions having a low sequence similarity with other Wnt proteins. As an illustration, the following regions of SEQ ID NO:2 provide suitable polypeptides for antibody production: amino acid residues 30 to 42, amino acid residues 44 to 62, amino acid residues 69 to 97, amino acid residues 101 to 111, amino acid residues 120 to 138, amino acid residues 143 to 177, amino acid residues 184 to 215, amino acid residues 217 to 231, amino acid residues 217 to 248, amino acid residues 231 to 248, amino acid residues 276 to 297, amino acid residues 321 to 345, amino acid residues 345 to 415, and amino acid residues 321 to 415.

Anti-Zwnt3 antibodies can be produced using antigenic Zwnt3 epitope-bearing peptides and polypeptides. Antigenic epitope-bearing peptides and polypeptides of the present invention contain a sequence of at least nine, or between 15 to about 30 amino acids contained within SEQ ID NO:2. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of the invention, containing from 30 to 50 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are useful for inducing antibodies that bind with Zwnt3. It is desirable that the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (*i.e.*, the sequence includes relatively hydrophilic residues, while hydrophobic residues are preferably avoided). Moreover, amino acid sequences containing proline residues may be also be desirable for antibody production.

Polyclonal antibodies to recombinant Zwnt3 protein or to Zwnt3 isolated from natural sources can be prepared using methods well-known to those of skill in the art. For example, Busse and Séguin, *Biochem. Mol. Biol. Int.* 30:607 (1993), describe the production of polyclonal antibodies against a synthetic peptide corresponding to a surface-exposed epitope of a Wnt-1 homologue. Antibodies can also be generated using a Zwnt3-glutathione transferase fusion protein, which is similar to a method described

by Burrus and McMahon, *Exp. Cell. Res.* 220:363 (1995). General methods for producing polyclonal antibodies are described, for example, by Green *et al.*, "Production of Polyclonal Antisera," in *Immunochemical Protocols* (Manson, ed.), pages 1-5 (Humana Press 1992), and Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 15 (Oxford University Press 1995).

The immunogenicity of a Zwn3 polypeptide can be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of Zwn3 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "haptene-like," such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

Although polyclonal antibodies are typically raised in animals such as horse, cow, dog, chicken, rat, mouse, rabbit, goat, guinea pig, or sheep, an anti-Zwn3 antibody of the present invention may also be derived from a subhuman primate antibody. General techniques for raising diagnostically and therapeutically useful antibodies in baboons may be found, for example, in Goldenberg *et al.*, international patent publication No. WO 91/11465, and in Losman *et al.*, *Int. J. Cancer* 46:310 (1990).

Alternatively, monoclonal anti-Zwn3 antibodies can be generated. Rodent monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art (see, for example, Kohler *et al.*, *Nature* 256:495 (1975), Coligan *et al.* (eds.), *Current Protocols in Immunology*, Vol. 1, pages 2.5.1-2.6.7 (John Wiley & Sons 1991) ["Coligan"], Picksley *et al.*, "Production of monoclonal antibodies against proteins expressed in *E. coli*," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 93 (Oxford University Press 1995)).

Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising a Zwn3 gene product, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

In addition, an anti-Zwnt3 antibody of the present invention may be derived from a human monoclonal antibody. Human monoclonal antibodies are obtained from transgenic mice that have been engineered to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described, for example, by Green *et al.*, *Nature Genet.* 7:13 (1994), Lonberg *et al.*, *Nature* 368:856 (1994), and Taylor *et al.*, *Int. Immun.* 6:579 (1994).

Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography (see, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3; Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in *Methods in Molecular Biology*, Vol. 10, pages 79-104 (The Humana Press, Inc. 1992)).

For particular uses, it may be desirable to prepare fragments of anti-Zwnt3 antibodies. Such antibody fragments can be obtained, for example, by proteolytic hydrolysis of the antibody. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. As an illustration, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent to produce 3.5S Fab' monovalent fragments. Optionally, the cleavage reaction can be performed using a blocking group for the sulfhydryl groups that result from cleavage of disulfide linkages. As an alternative, an enzymatic cleavage using pepsin produces two monovalent Fab fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent No. 4,331,647, Nisonoff *et al.*, *Arch Biochem. Biophys.* 89:230 (1960), Porter, *Biochem. J.* 73:119 (1959), Edelman *et al.*, in *Methods in Enzymology Vol. 1*, page 422 (Academic Press 1967), and by Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise an association of V_H and V_L chains. This association can be noncovalent, as described by Inbar *et al.*, *Proc. Nat'l Acad. Sci.*

USA 69:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (see, for example, Sandhu, *Crit. Rev. Biotech.* 12:437 (1992)).

5 The Fv fragments may comprise V_H and V_L chains which are connected by a peptide linker. These single-chain antigen binding proteins (scFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains which are connected by an oligonucleotide. The structural gene is inserted into an expression vector which is subsequently introduced into a host cell, such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing scFvs are described, for example, by Whitlōw *et al.*, *Methods: A Companion to Methods in Enzymology* 2:97 (1991) (also see, Bird *et al.*, *Science* 242:423 (1988), Ladner *et al.*, U.S. Patent No. 4,946,778, Pack *et al.*, *Bio/Technology* 11:1271 (1993), and Sandhu, *supra*).

15 As an illustration, a scFV can be obtained by exposing lymphocytes to Zwnt3 polypeptide *in vitro*, and selecting antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled Zwnt3 protein or peptide). Genes encoding polypeptides having potential Zwnt3 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be
20 obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide
25 display libraries are known in the art (Ladner *et al.*, U.S. Patent No. 5,223,409, Ladner *et al.*, U.S. Patent No. 4,946,778, Ladner *et al.*, U.S. Patent No. 5,403,484, Ladner *et al.*, U.S. Patent No. 5,571,698, and Kay *et al.*, *Phage Display of Peptides and Proteins* (Academic Press, Inc. 1996)) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from CLONTECH
30 Laboratories, Inc. (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA), and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the Zwnt3 sequences disclosed herein to identify proteins which bind to Zwnt3.

35 Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction

to synthesize the variable region from RNA of antibody-producing cells (see, for example, Larrick *et al.*, *Methods: A Companion to Methods in Enzymology* 2:106 (1991), Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in *Monoclonal Antibodies: Production, Engineering and Clinical Application*, Ritter *et al.* (eds.), page 166 (Cambridge University Press 1995), and Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, Birch *et al.*, (eds.), page 137 (Wiley-Liss, Inc. 1995)).

Alternatively, an anti-Zwnt3 antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementary determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain. Typical residues of human antibodies are then substituted in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:3833 (1989). Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature* 321:522 (1986), Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* 89:4285 (1992), Sandhu, *Crit. Rev. Biotech.* 12:437 (1992), Singer *et al.*, *J. Immun.* 150:2844 (1993), Sudhir (ed.), *Antibody Engineering Protocols* (Humana Press, Inc. 1995), Kelley, "Engineering Therapeutic Antibodies," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 399-434 (John Wiley & Sons, Inc. 1996), and by Queen *et al.*, U.S. Patent No. 5,693,762 (1997).

Polyclonal anti-idiotypic antibodies can be prepared by immunizing animals with anti-Zwnt3 antibodies or antibody fragments, using standard techniques. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in *Methods In Molecular Biology: Immunochemical Protocols*, Manson (ed.), pages 1-12 (Humana Press 1992). Also, see Coligan at pages 2.4.1-2.4.7. Alternatively, monoclonal anti-idiotypic antibodies can be prepared using anti-Zwnt3 antibodies or antibody fragments as immunogens with the techniques, described above. As another alternative, humanized anti-idiotypic antibodies or subhuman primate anti-idiotypic antibodies can be prepared using the above-described techniques. Methods for producing anti-idiotypic antibodies are described, for example, by Irie, U.S. Patent No. 5,208,146, Greene, *et al.*, U.S. Patent No. 5,637,677, and Varthakavi and Minocha, *J. Gen. Virol.* 77:1875 (1996).

Anti-idiotypic Zwnt3 antibodies, as well as Zwnt3 polypeptides, can be used to identify and to isolate Zwnt3 receptors. For example, proteins and peptides of

the present invention can be immobilized on a column and used to bind receptor proteins from membrane preparations that are run over the column (Hermanson *et al.* (eds.), *Immobilized Affinity Ligand Techniques*, pages 195-202 (Academic Press 1992)). Radiolabeled or affinity labeled Zwnt3 polypeptides can also be used to
5 identify or to localize Zwnt3 receptors in a biological sample (see, for example, Deutscher (ed.), *Methods in Enzymol.*, vol. 182, pages 721-37 (Academic Press 1990); Brunner *et al.*, *Ann. Rev. Biochem.* 62:483 (1993); Fedan *et al.*, *Biochem. Pharmacol.* 33:1167 (1984)). Also see, Varthakavi and Minocha, *J. Gen. Virol.* 77:1875 (1996), who describe the use of anti-idiotypic antibodies for receptor identification.

10

9. Use of Zwnt3 Nucleotide Sequences to Detect Zwnt3 Gene Expression and to Examine Zwnt3 Gene Structure

Nucleic acid molecules can be used to detect the expression of a Zwnt3 gene in a biological sample. Such probe molecules include double-stranded nucleic
15 acid molecules comprising the nucleotide sequence of SEQ ID NO:1, or a fragment thereof, as well as single-stranded nucleic acid molecules having the complement of the nucleotide sequence of SEQ ID NO:1, or a fragment thereof. Probe molecules may be DNA, RNA, oligonucleotides, and the like.

Certain probes bind with regions of a *Zwnt3* gene that have a low
20 sequence similarity to comparable regions in other Wnt proteins. For example, suitable probes include portions of the following nucleotide sequences of SEQ ID NO:1, or complements thereof: nucleotides 694 to 741, and nucleotides 961 to 1245. As used herein, the term "portion" refers to at least eight nucleotides to at least 20 or more nucleotides.

25 In a basic assay, a single-stranded probe molecule is incubated with RNA, isolated from a biological sample, under conditions of temperature and ionic strength that promote base pairing between the probe and target Zwnt3 RNA species. After separating unbound probe from hybridized molecules, the amount of hybrids is detected.

30 Well-established hybridization methods of RNA detection include northern analysis and dot/slot blot hybridization (see, for example, Ausubel (1995) at pages 4-1 to 4-27, and Wu *et al.* (eds.), "Analysis of Gene Expression at the RNA Level," in *Methods in Gene Biotechnology*, pages 225-239 (CRC Press, Inc. 1997)). Nucleic acid probes can be detectably labeled with radioisotopes such as ³²P or ³⁵S.
35 Alternatively, Zwnt3 RNA can be detected with a nonradioactive hybridization method (see, for example, Isaac (ed.), *Protocols for Nucleic Acid Analysis by Nonradioactive*

Probes (Humana Press, Inc. 1993)). Typically, nonradioactive detection is achieved by enzymatic conversion of chromogenic or chemiluminescent substrates. Illustrative nonradioactive moieties include biotin, fluorescein, and digoxigenin.

5 Zwnt3 oligonucleotide probes are also useful for *in vivo* diagnosis. As an illustration, ¹⁸F-labeled oligonucleotides can be administered to a subject and visualized by positron emission tomography (Tavitian *et al.*, *Nature Medicine* 4:467 (1998)).

10 Numerous diagnostic procedures take advantage of the polymerase chain reaction (PCR) to increase sensitivity of detection methods. Standard techniques for performing PCR are well-known (see, generally, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), White (ed.), *PCR Protocols: Current Methods and Applications* (Humana Press, Inc. 1993), Cotter (ed.), *Molecular Diagnosis of Cancer* (Humana Press, Inc. 1996), Hanausek and Walaszek (eds.), *Tumor Marker Protocols* (Humana Press, Inc. 1998), Lo (ed.), *Clinical Applications of PCR* (Humana Press, Inc. 1998), and Meltzer (ed.), *PCR in Bioanalysis* (Humana Press, Inc. 15 1998)).

 Certain PCR primers are designed to amplify a portion of the *Zwnt3* gene that has a low sequence similarity to a comparable region in other Wnt sequences. As an illustration, suitable primers are designed to amplify regions within nucleotides 694 to 741, and nucleotides 961 to 1245 of SEQ ID NO:1.

20 One variation of PCR for diagnostic assays is reverse transcriptase-PCR (RT-PCR). In the RT-PCR technique, RNA is isolated from a biological sample, reverse transcribed to cDNA, and the cDNA is incubated with *Zwnt3* primers (see, for example, Wu *et al.* (eds.), "Rapid Isolation of Specific cDNAs or Genes by PCR," in *Methods in Gene Biotechnology*, pages 15-28 (CRC Press, Inc. 1997)). PCR is then 25 performed and the products are analyzed using standard techniques.

 As an illustration, RNA is isolated from biological sample using, for example, the guanidinium-thiocyanate cell lysis procedure described above. Alternatively, a solid-phase technique can be used to isolate mRNA from a cell lysate. A reverse transcription reaction can be primed with the isolated RNA using random 30 oligonucleotides, short homopolymers of dT, or *Zwnt3* anti-sense oligomers. Oligo-dT primers offer the advantage that various mRNA nucleotide sequences are amplified that can provide control target sequences. *Zwnt3* sequences are amplified by the polymerase chain reaction using two flanking oligonucleotide primers that are typically 20 bases in length.

35 PCR amplification products can be detected using a variety of approaches. For example, PCR products can be fractionated by gel electrophoresis, and visualized by ethidium bromide staining. Alternatively, fractionated PCR products can

be transferred to a membrane, hybridized with a detectably-labeled *Zwnt3* probe, and examined by autoradiography. Additional alternative approaches include the use of digoxigenin-labeled deoxyribonucleic acid triphosphates to provide chemiluminescence detection, and the C-TRAK colorimetric assay.

5 Another approach for detection of *Zwnt3* expression is cycling probe technology (CPT), in which a single-stranded DNA target binds with an excess of DNA-RNA-DNA chimeric probe to form a complex, the RNA portion is cleaved with RNAase H, and the presence of cleaved chimeric probe is detected (see, for example, Beggs *et al.*, *J. Clin. Microbiol.* 34:2985 (1996), Bekkaoui *et al.*, *Biotechniques* 20:240
10 (1996)). Alternative methods for detection of *Zwnt3* sequences can utilize approaches such as nucleic acid sequence-based amplification (NASBA), cooperative amplification of templates by cross-hybridization (CATCH), and the ligase chain reaction (LCR) (see, for example, Marshall *et al.*, U.S. Patent No. 5,686,272 (1997), Dyer *et al.*, *J. Virol. Methods* 60:161 (1996), Ehricht *et al.*, *Eur. J. Biochem.* 243:358 (1997), and Chadwick
15 *et al.*, *J. Virol. Methods* 70:59 (1998)). Other standard methods are known to those of skill in the art.

Zwnt3 probes and primers can also be used to detect and to localize *Zwnt3* gene expression in tissue samples. Methods for such *in situ* hybridization are well-known to those of skill in the art (see, for example, Choo (ed.), *In Situ Hybridization Protocols* (Humana Press, Inc. 1994), Wu *et al.* (eds.), "Analysis of Cellular DNA or Abundance of mRNA by Radioactive *In Situ* Hybridization (RISH)," in *Methods in Gene Biotechnology*, pages 259-278 (CRC Press, Inc. 1997), and Wu *et al.* (eds.), "Localization of DNA or Abundance of mRNA by Fluorescence *In Situ* Hybridization (RISH)," in *Methods in Gene Biotechnology*, pages 279-289 (CRC Press, Inc. 1997)). Various additional diagnostic approaches are well-known to those of skill
20 in the art (see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Coleman and Tsongalis, *Molecular Diagnostics* (Humana Press, Inc. 1996), and Elles, *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc., 1996)). Suitable test samples include blood, urine, saliva, tissue biopsy, and
25 autopsy material.
30

Nucleic acid molecules comprising *Zwnt3* nucleotide sequences can be used to determine whether a subject's chromosomes contain a mutation in the *Zwnt3* gene. Detectable chromosomal aberrations at the *Zwnt3* gene locus include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. Of particular interest are genetic alterations that inactivate
35 a *Zwnt3* gene.

Aberrations associated with a *Zwnt3* locus can be detected using nucleic acid molecules of the present invention by employing molecular genetic techniques, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, amplification-refractory mutation system analysis (ARMS), single-strand conformation polymorphism (SSCP) detection, RNase cleavage methods, denaturing gradient gel electrophoresis, fluorescence-assisted mismatch analysis (FAMA), and other genetic analysis techniques known in the art (see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Marian, *Chest* 108:255 (1995), Coleman and Tsongalis, *Molecular Diagnostics* (Human Press, Inc. 1996), Elles (ed.) *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc. 1996), Landegren (ed.), *Laboratory Protocols for Mutation Detection* (Oxford University Press 1996), Birren *et al.* (eds.), *Genome Analysis, Vol. 2: Detecting Genes* (Cold Spring Harbor Laboratory Press 1998), Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics* (John Wiley & Sons 1998), and Richards and Ward, "Molecular Diagnostic Testing," in *Principles of Molecular Medicine*, pages 83-88 (Humana Press, Inc. 1998)).

The chromosomal location of the *Zwnt3* gene can be determined using radiation hybrid mapping, which is a somatic cell genetic technique developed for constructing high-resolution, contiguous maps of mammalian chromosomes (Cox *et al.*, *Science* 250:245 (1990)). Partial or full knowledge of a gene's sequence allows one to design PCR primers suitable for use with chromosomal radiation hybrid mapping panels. Radiation hybrid mapping panels are commercially available which cover the entire human genome, such as the Stanford G3 RH Panel and the GeneBridge 4 RH Panel (Research Genetics, Inc., Huntsville, AL). These panels enable rapid, PCR-based chromosomal localizations and ordering of genes, sequence-tagged sites, and other nonpolymorphic and polymorphic markers within a region of interest. This includes establishing directly proportional physical distances between newly discovered genes of interest and previously mapped markers.

The protein truncation test is also useful for detecting the inactivation of a gene in which translation-terminating mutations produce only portions of the encoded protein (see, for example, Stoppa-Lyonnet *et al.*, *Blood* 91:3920 (1998)). According to this approach, RNA is isolated from a biological sample, and used to synthesize cDNA. PCR is then used to amplify the *Zwnt3* target sequence and to introduce an RNA polymerase promoter, a translation initiation sequence, and an in-frame ATG triplet. PCR products are transcribed using an RNA polymerase, and the transcripts are translated *in vitro* with a T7-coupled reticulocyte lysate system. The translation products are then fractionated by SDS-PAGE to determine the lengths of the translation

products. The protein truncation test is described, for example, by Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics*, pages 9.11.1 - 9.11.18 (John Wiley & Sons 1998).

5 The present invention also contemplates kits for performing a diagnostic assay for *Zwnt3* gene expression or to analyze the *Zwnt3* locus of a subject. Such kits comprise nucleic acid probes, such as double-stranded nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO:1, or a fragment thereof, as well as single-stranded nucleic acid molecules having the complement of the nucleotide sequence of SEQ ID NO:1, or a fragment thereof. Illustrative fragments reside within 10 nucleotides 961 to 1245, and nucleotides 694 to 741 of SEQ ID NO:1. Probe molecules may be DNA, RNA, oligonucleotides, and the like. Kits may comprise nucleic acid primers for performing PCR.

Such a kit can contain all the necessary elements to perform a nucleic acid diagnostic assay described above. A kit will comprise at least one container 15 comprising a *Zwnt3* probe or primer. The kit may also comprise a second container comprising one or more reagents capable of indicating the presence of *Zwnt3* sequences. Examples of such indicator reagents include detectable labels such as radioactive labels, fluorochromes, chemiluminescent agents, and the like. A kit may also comprise a means for conveying to the user that the *Zwnt3* probes and primers are 20 used to detect *Zwnt3* gene expression. For example, written instructions may state that the enclosed nucleic acid molecules can be used to detect either a nucleic acid molecule that encodes *Zwnt3*, or a nucleic acid molecule having a nucleotide sequence that is complementary to a *Zwnt3*-encoding nucleotide sequence, or to analyze chromosomal sequences associated with the *Zwnt3* locus. The written material can be applied directly 25 to a container, or the written material can be provided in the form of a packaging insert.

10. **Use of Anti-Zwnt3 Antibodies to Detect Zwnt3 Protein**

The present invention contemplates the use of anti-Zwnt3 antibodies to screen biological samples *in vitro* for the presence of Zwnt3. In one type of *in vitro* assay, 30 anti-Zwnt3 antibodies are used in liquid phase. For example, the presence of Zwnt3 in a biological sample can be tested by mixing the biological sample with a trace amount of labeled Zwnt3 and an anti-Zwnt3 antibody under conditions that promote binding between Zwnt3 and its antibody. Complexes of Zwnt3 and anti-Zwnt3 in the sample can be separated from the reaction mixture by contacting the complex with an immobilized 35 protein which binds with the antibody, such as an Fc antibody or *Staphylococcus* protein A. The concentration of Zwnt3 in the biological sample will be inversely proportional to

the amount of labeled Zwnt3 bound to the antibody and directly related to the amount of free labeled Zwnt3.

Alternatively, *in vitro* assays can be performed in which anti-Zwnt3 antibody is bound to a solid-phase carrier. For example, antibody can be attached to a polymer, such as aminodextran, in order to link the antibody to an insoluble support such as a polymer-coated bead, a plate or a tube. Other suitable *in vitro* assays will be readily apparent to those of skill in the art.

In another approach, anti-Zwnt3 antibodies can be used to detect Zwnt3 in tissue sections prepared from a biopsy specimen. Such immunochemical detection can be used to determine the relative abundance of Zwnt3 and to determine the distribution of Zwnt3 in the examined tissue. General immunochemistry techniques are well established (see, for example, Ponder, "Cell Marking Techniques and Their Application," in *Mammalian Development: A Practical Approach*, Monk (ed.), pages 115-38 (IRL Press 1987), Coligan at pages 5.8.1-5.8.8, Ausubel (1995) at pages 14.6.1 to 14.6.13 (Wiley Interscience 1990), and Manson (ed.), *Methods In Molecular Biology, Vol.10: Immunochemical Protocols* (The Humana Press, Inc. 1992)).

Immunochemical detection can be performed by contacting a biological sample with an anti-Zwnt3 antibody, and then contacting the biological sample with a detectably labeled molecule which binds to the antibody. For example, the detectably labeled molecule can comprise an antibody moiety that binds to anti-Zwnt3 antibody. Alternatively, the anti-Zwnt3 antibody can be conjugated with avidin/streptavidin (or biotin) and the detectably labeled molecule can comprise biotin (or avidin/streptavidin). Numerous variations of this basic technique are well-known to those of skill in the art.

Alternatively, an anti-Zwnt3 antibody can be conjugated with a detectable label to form an anti-Zwnt3 immunoconjugate. Suitable detectable labels include, for example, a radioisotope, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label or colloidal gold. Methods of making and detecting such detectably-labeled immunoconjugates are well-known to those of ordinary skill in the art, and are described in more detail below.

The detectable label can be a radioisotope that is detected by autoradiography. Isotopes that are particularly useful for the purpose of the present invention are ^3H , ^{125}I , ^{131}I , ^{35}S and ^{14}C .

Anti-Zwnt3 immunoconjugates can also be labeled with a fluorescent compound. The presence of a fluorescently-labeled antibody is determined by exposing the immunoconjugate to light of the proper wavelength and detecting the resultant fluorescence. Fluorescent labeling compounds include fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Alternatively, anti-Zwnt3 immunoconjugates can be detectably labeled by coupling an antibody component to a chemiluminescent compound. The presence of the chemiluminescent-tagged immunoconjugate is determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of chemiluminescent labeling compounds include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt and an oxalate ester.

Similarly, a bioluminescent compound can be used to label anti-Zwnt3 immunoconjugates of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Bioluminescent compounds that are useful for labeling include luciferin, luciferase and aequorin.

Alternatively, anti-Zwnt3 immunoconjugates can be detectably labeled by linking an anti-Zwnt3 antibody component to an enzyme. When the anti-Zwnt3-enzyme conjugate is incubated in the presence of the appropriate substrate, the enzyme moiety reacts with the substrate to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or visual means. Examples of enzymes that can be used to detectably label polyspecific immunoconjugates include β -galactosidase, glucose oxidase, peroxidase and alkaline phosphatase.

Those of skill in the art will know of other suitable labels which can be employed in accordance with the present invention. The binding of marker moieties to anti-Zwnt3 antibodies can be accomplished using standard techniques known to the art. Typical methodology in this regard is described by Kennedy *et al.*, *Clin. Chim. Acta* 70:1 (1976), Schurs *et al.*, *Clin. Chim. Acta* 81:1 (1977), Shih *et al.*, *Int'l J. Cancer* 46:1101 (1990), Stein *et al.*, *Cancer Res.* 50:1330 (1990), and Coligan, *supra*.

Moreover, the convenience and versatility of immunochemical detection can be enhanced by using anti-Zwnt3 antibodies that have been conjugated with avidin, streptavidin, and biotin (see, for example, Wilchek *et al.* (eds.), "Avidin-Biotin Technology," *Methods In Enzymology*, Vol. 184 (Academic Press 1990), and Bayer *et al.*, "Immunochemical Applications of Avidin-Biotin Technology," in *Methods In Molecular Biology*, Vol. 10, Manson (ed.), pages 149-162 (The Humana Press, Inc. 1992).

Methods for performing immunoassays are well-established. See, for example, Cook and Self, "Monoclonal Antibodies in Diagnostic Immunoassays," in *Monoclonal Antibodies: Production, Engineering, and Clinical Application*, Ritter and Ladyman (eds.), pages 180-208, (Cambridge University Press, 1995), Perry, "The Role of Monoclonal Antibodies in the Advancement of Immunoassay Technology," in *Monoclonal Antibodies: Principles and Applications*, Birch and Lennox (eds.), pages

107-120 (Wiley-Liss, Inc. 1995), and Diamandis, *Immunoassay* (Academic Press, Inc. 1996).

5 In a related approach, biotin- or FITC-labeled Zwnt3 can be used to identify cells that bind Zwnt3. Such can binding can be detected, for example, using flow cytometry.

10 The present invention also contemplates kits for performing an immunological diagnostic assay for *Zwnt3* gene expression. Such kits comprise at least one container comprising an anti-Zwnt3 antibody, or antibody fragment. A kit may also comprise a second container comprising one or more reagents capable of indicating the presence of Zwnt3 antibody or antibody fragments. Examples of such indicator reagents include detectable labels such as a radioactive label, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label, colloidal gold, and the like. A kit may also comprise a means for conveying to the user that Zwnt3 antibodies or antibody fragments are used to detect Zwnt3 protein. For example, written instructions may state that the enclosed antibody or antibody fragment can be used to detect Zwnt3. The written material can be applied directly to a container, or the written material can be provided in the form of a packaging insert.

11. *Therapeutic Uses of Polypeptides Having Zwnt3 Activity*

20 The present invention includes the use of proteins, polypeptides, and peptides having Zwnt3 activity (such as Zwnt3 polypeptides, anti-idiotypic anti-Zwnt3 antibodies, and Zwnt3 fusion proteins) to a subject who lacks an adequate amount of this Wnt polypeptide. These molecules can be administered to any subject in need of treatment, and the present invention contemplates both veterinary and human therapeutic uses. Illustrative subjects include mammalian subjects, such as farm animals, domestic animals, and human patients.

30 Generally, the dosage of administered polypeptide, protein or peptide will vary depending upon such factors as the subject's age, weight, height, sex, general medical condition and previous medical history. Typically, it is desirable to provide the recipient with a dosage of a molecule having Zwnt3 activity which is in the range of from about 1 pg/kg to 10 mg/kg (amount of agent/body weight of subject), although a lower or higher dosage also may be administered as circumstances dictate.

35 Administration of a molecule having Zwnt3 activity to a subject can be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional catheter, or by direct intralesional injection.

When administering therapeutic proteins by injection, the administration may be by continuous infusion or by single or multiple boluses.

5 A pharmaceutical composition comprising a protein, polypeptide, or peptide having Zwnt3 activity can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the therapeutic proteins are combined in a mixture with a pharmaceutically acceptable carrier. A composition is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient patient. Sterile phosphate-buffered saline is one example of a pharmaceutically acceptable carrier. Other suitable carriers are well-known to those in the art. See, for example, Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company 1995).

For purposes of therapy, molecules having Zwnt3 activity and a pharmaceutically acceptable carrier are administered to a patient in a therapeutically effective amount. A combination of a protein, polypeptide, or peptide having Zwnt3 activity and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

A pharmaceutical composition comprising molecules having Zwnt3 activity can be furnished in liquid form, or in solid form. Liquid forms, including liposome-encapsulated formulations, are illustrated by injectable solutions and oral suspensions. Exemplary solid forms include capsules, tablets, and controlled-release forms, such as a miniosmotic pump or an implant. Other dosage forms can be devised by those skilled in the art, as shown, for example, by Ansel and Popovich, *Pharmaceutical Dosage Forms and Drug Delivery Systems*, 5th Edition (Lea & Febiger 1990), Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company 1995), and by Ranade and Hollinger, *Drug Delivery Systems* (CRC Press 1996).

As an illustration, Zwnt3 pharmaceutical compositions may be supplied as a kit comprising a container that comprises Zwnt3. Zwnt3 can be provided in the form of an injectable solution for single or multiple doses, or as a sterile powder that will be reconstituted before injection. Such a kit may further comprise written information on indications and usage of the pharmaceutical composition. Moreover, such information may include a statement that the Zwnt3 composition is contraindicated in patients with known hypersensitivity to Zwnt3.

12. Therapeutic Uses of *Zwnt3* Nucleotide Sequences

The present invention includes the use of *Zwnt3* nucleotide sequences to provide *Zwnt3* to a subject in need of such treatment. In addition, a therapeutic expression vector can be provided that inhibits *Zwnt3* gene expression, such as an anti-sense molecule, a ribozyme, or an external guide sequence molecule.

There are numerous approaches to introduce a *Zwnt3* gene to a subject, including the use of recombinant host cells that express *Zwnt3*, delivery of naked nucleic acid encoding *Zwnt3*, use of a cationic lipid carrier with a nucleic acid molecule that encodes *Zwnt3*, and the use of viruses that express *Zwnt3*, such as recombinant retroviruses, recombinant adeno-associated viruses, recombinant adenoviruses, and recombinant Herpes simplex viruses [HSV] (see, for example, Mulligan, *Science* 260:926 (1993), Rosenberg *et al.*, *Science* 242:1575 (1988), LaSalle *et al.*, *Science* 259:988 (1993), Wolff *et al.*, *Science* 247:1465 (1990), Breakfield and Deluca, *The New Biologist* 3:203 (1991)). In an *ex vivo* approach, for example, cells are isolated from a subject, transfected with a vector that expresses a *Zwnt3* gene, and then transplanted into the subject.

In order to effect expression of a *Zwnt3* gene, an expression vector is constructed in which a nucleotide sequence encoding a *Zwnt3* gene is operably linked to a core promoter, and optionally a regulatory element, to control gene transcription. The general requirements of an expression vector are described above.

Alternatively, a *Zwnt3* gene can be delivered using recombinant viral vectors, including for example, adenoviral vectors (*e.g.*, Kass-Eisler *et al.*, *Proc. Nat'l Acad. Sci. USA* 90:11498 (1993), Kolls *et al.*, *Proc. Nat'l Acad. Sci. USA* 91:215 (1994), Li *et al.*, *Hum. Gene Ther.* 4:403 (1993), Vincent *et al.*, *Nat. Genet.* 5:130 (1993), and Zabner *et al.*, *Cell* 75:207 (1993)), adenovirus-associated viral vectors (Flotte *et al.*, *Proc. Nat'l Acad. Sci. USA* 90:10613 (1993)), alphaviruses such as Semliki Forest Virus and Sindbis Virus (Hertz and Huang, *J. Vir.* 66:857 (1992), Raju and Huang, *J. Vir.* 65:2501 (1991), and Xiong *et al.*, *Science* 243:1188 (1989)), herpes viral vectors (*e.g.*, U.S. Patent Nos. 4,769,331, 4,859,587, 5,288,641 and 5,328,688), parvovirus vectors (Koering *et al.*, *Hum. Gene Therap.* 5:457 (1994)), pox virus vectors (Ozaki *et al.*, *Biochem. Biophys. Res. Comm.* 193:653 (1993), Panicali and Paoletti, *Proc. Nat'l Acad. Sci. USA* 79:4927 (1982)), pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:317 (1989), and Flexner *et al.*, *Ann. N.Y. Acad. Sci.* 569:86 (1989)), and retroviruses (*e.g.*, Baba *et al.*, *J. Neurosurg* 79:729 (1993), Ram *et al.*, *Cancer Res.* 53:83 (1993), Takamiya *et al.*, *J. Neurosci. Res* 33:493 (1992), Vile and Hart, *Cancer Res.* 53:962 (1993), Vile and Hart, *Cancer Res.* 53:3860 (1993), and Anderson *et al.*, U.S. Patent No. 5,399,346). Within

various embodiments, either the viral vector itself, or a viral particle which contains the viral vector may be utilized in the methods and compositions described below.

As an illustration of one system, adenovirus, a double-stranded DNA virus, is a well-characterized gene transfer vector for delivery of a heterologous nucleic acid molecule (for a review, see Becker *et al.*, *Meth. Cell Biol.* 43:161 (1994); Douglas and Curiel, *Science & Medicine* 4:44 (1997)). The adenovirus system offers several advantages including: (i) the ability to accommodate relatively large DNA inserts, (ii) the ability to be grown to high-titer, (iii) the ability to infect a broad range of mammalian cell types, and (iv) the ability to be used with many different promoters including ubiquitous, tissue specific, and regulatable promoters. In addition, adenoviruses can be administered by intravenous injection, because the viruses are stable in the bloodstream.

Using adenovirus vectors where portions of the adenovirus genome are deleted, inserts are incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene is deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell. When intravenously administered to intact animals, adenovirus primarily targets the liver. Although an adenoviral delivery system with an E1 gene deletion cannot replicate in the host cells, the host's tissue will express and process an encoded heterologous protein. Host cells will also secrete the heterologous protein if the corresponding gene includes a secretory signal sequence. Secreted proteins will enter the circulation from tissue that expresses the heterologous gene (*e.g.*, the highly vascularized liver).

Moreover, adenoviral vectors containing various deletions of viral genes can be used to reduce or eliminate immune responses to the vector. Such adenoviruses are E1-deleted, and in addition, contain deletions of E2A or E4 (Lusky *et al.*, *J. Virol.* 72:2022 (1998); Raper *et al.*, *Human Gene Therapy* 9:671 (1998)). The deletion of E2b has also been reported to reduce immune responses (Amalfitano *et al.*, *J. Virol.* 72:926 (1998)). By deleting the entire adenovirus genome, very large inserts of heterologous DNA can be accommodated. Generation of so called "gutless" adenoviruses, where all viral genes are deleted, are particularly advantageous for insertion of large inserts of heterologous DNA (for a review, see Yeh. and Perricaudet, *FASEB J.* 11:615 (1997)).

High titer stocks of recombinant viruses capable of expressing a therapeutic gene can be obtained from infected mammalian cells using standard methods. For example, recombinant HSV can be prepared in Vero cells, as described by Brandt *et al.*, *J. Gen. Virol.* 72:2043 (1991), Herold *et al.*, *J. Gen. Virol.* 75:1211 (1994), Visalli and Brandt, *Virology* 185:419 (1991), Grau *et al.*, *Invest. Ophthalmol.*

Vis. Sci. 30:2474 (1989), Brandt *et al.*, *J. Virol. Meth.* 36:209 (1992), and by Brown and MacLean (eds.), *HSV Virus Protocols* (Humana Press 1997).

Alternatively, an expression vector comprising a *Zwnt3* gene can be introduced into a subject's cells by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner *et al.*, *Proc. Nat'l Acad. Sci. USA* 84:7413 (1987); Mackey *et al.*, *Proc. Nat'l Acad. Sci. USA* 85:8027 (1988)). The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Liposomes can be used to direct transfection to particular cell types, which is particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (*e.g.*, hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

Electroporation is another alternative mode of administration of a *Zwnt3* nucleic acid molecules. For example, Aihara and Miyazaki, *Nature Biotechnology* 16:867 (1998), have demonstrated the use of *in vivo* electroporation for gene transfer into muscle.

In an alternative approach to gene therapy, a therapeutic gene may encode a *Zwnt3* anti-sense RNA that inhibits the expression of *Zwnt3*. Methods of preparing anti-sense Wnt constructs are known to those in the art. See, for example, Erickson *et al.*, *Dev. Genet.* 14:274 (1993) [transgenic mice], Augustine *et al.*, *Dev. Genet.* 14:500 (1993) [murine whole embryo culture], and Olson and Gibo, *Exp. Cell Res.* 241:134 (1998) [cultured cells]. Suitable sequences for *Zwnt3* anti-sense molecules can be derived from the nucleotide sequences of *Zwnt3* disclosed herein.

Alternatively, an expression vector can be constructed in which a regulatory element is operably linked to a nucleotide sequence that encodes a ribozyme. Ribozymes can be designed to express endonuclease activity that is directed to a certain target sequence in a mRNA molecule (see, for example, Draper and Macejak, U.S. Patent No. 5,496,698, McSwiggen, U.S. Patent No. 5,525,468, Chowrira and McSwiggen, U.S. Patent No. 5,631,359, and Robertson and Goldberg, U.S. Patent No. 5,225,337). In the context of the present invention, ribozymes include nucleotide sequences that bind with *Zwnt3* mRNA.

In another approach, expression vectors can be constructed in which a regulatory element directs the production of RNA transcripts capable of promoting RNase P-mediated cleavage of mRNA molecules that encode a *Zwnt3* gene. According to this approach, an external guide sequence can be constructed for directing the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, which is subsequently

cleaved by the cellular ribozyme (see, for example, Altman *et al.*, U.S. Patent No. 5,168,053, Yuan *et al.*, *Science* 263:1269 (1994), Pace *et al.*, international publication No. WO 96/18733, George *et al.*, international publication No. WO 96/21731, and Werner *et al.*, international publication No. WO 97/33991). Preferably, the external
5 guide sequence comprises a ten to fifteen nucleotide sequence complementary to Zwn3 mRNA, and a 3'-NCCA nucleotide sequence, wherein N is preferably a purine. The external guide sequence transcripts bind to the targeted mRNA species by the formation of base pairs between the mRNA and the complementary external guide sequences, thus promoting cleavage of mRNA by RNase P at the nucleotide located at the 5'-side of the
10 base-paired region.

In general, the dosage of a composition comprising a therapeutic vector having a Zwn3 nucleotide acid sequence, such as a recombinant virus, will vary depending upon such factors as the subject's age, weight, height, sex, general medical condition and previous medical history. Suitable routes of administration of therapeutic
15 vectors include intravenous injection, intraarterial injection, intraperitoneal injection, intramuscular injection, intratumoral injection, and injection into a cavity that contains a tumor.

A composition comprising viral vectors, non-viral vectors, or a combination of viral and non-viral vectors of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby
20 vectors or viruses are combined in a mixture with a pharmaceutically acceptable carrier. As noted above, a composition, such as phosphate-buffered saline is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient subject. Other suitable carriers are well-known to those in the art (see, for
25 example, *Remington's Pharmaceutical Sciences, 19th Ed.* (Mack Publishing Co. 1995), and *Gilman's the Pharmacological Basis of Therapeutics, 7th Ed.* (MacMillan Publishing Co. 1985)).

For purposes of therapy, a therapeutic gene expression vector, or a recombinant virus comprising such a vector, and a pharmaceutically acceptable carrier
30 are administered to a subject in a therapeutically effective amount. A combination of an expression vector (or virus) and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient subject.

35 When the subject treated with a therapeutic gene expression vector or a recombinant virus is a human, then the therapy is preferably somatic cell gene therapy. That is, the preferred treatment of a human with a therapeutic gene expression vector or

a recombinant virus does not entail introducing into cells a nucleic acid molecule that can form part of a human germ line and be passed onto successive generations (*i.e.*, human germ line gene therapy).

CLAIMS

What is claimed is:

1. An isolated polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2, wherein the isolated polypeptide specifically binds with an antibody that specifically binds with a polypeptide consisting of the amino acid sequence of SEQ ID NO:2.
2. The isolated polypeptide of claim 1, wherein the isolated polypeptide has an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:2.
3. The isolated polypeptide of claim 1, wherein the isolated polypeptide comprises the amino acid sequence of SEQ ID NO:2.
4. An isolated polypeptide comprising an amino acid sequence of at least 15 contiguous amino acids of an amino acid sequence selected from the group consisting of: amino acid residues 44 to 62 of SEQ ID NO:2, amino acid residues 69 to 97 of SEQ ID NO:2, amino acid residues 120 to 138 of SEQ ID NO:2, amino acid residues 143 to 177 of SEQ ID NO:2, amino acid residues 184 to 215 of SEQ ID NO:2, amino acid residues 217 to 231 of SEQ ID NO:2, amino acid residues 217 to 248 of SEQ ID NO:2, amino acid residues 231 to 248 of SEQ ID NO:2, amino acid residues 276 to 297 of SEQ ID NO:2, amino acid residues 321 to 345 of SEQ ID NO:2, amino acid residues 345 to 415 of SEQ ID NO:2, and amino acid residues 321 to 415 of SEQ ID NO:2.
5. The isolated polypeptide of claim 4, comprising an amino acid sequence selected from the group consisting of: amino acid residues 30 to 42 of SEQ ID NO:2, amino acid residues 44 to 62 of SEQ ID NO:2, amino acid residues 69 to 97 of SEQ ID NO:2, amino acid residues 101 to 111 of SEQ ID NO:2, amino acid residues 120 to 138 of SEQ ID NO:2, amino acid residues 143 to 177 of SEQ ID NO:2, amino acid residues 184 to 215 of SEQ ID NO:2, amino acid residues 217 to 231 of SEQ ID NO:2, amino acid residues 217 to 248 of SEQ ID NO:2, amino acid residues 231 to 248 of SEQ ID NO:2, amino acid residues 276 to 297 of SEQ ID NO:2, amino acid residues 321 to 345 of SEQ ID NO:2, amino acid residues 345 to 415 of SEQ ID NO:2, and amino acid residues 321 to 415 of SEQ ID NO:2.

6. An isolated nucleic acid molecule, wherein the nucleic acid molecule is selected from the group consisting of: (a) a nucleic acid molecule encoding the amino acid sequence of SEQ ID NO:2, and (b) a nucleic acid molecule that remains hybridized following stringent wash conditions to a nucleic acid molecule consisting of a nucleotide sequence selected from the group consisting of: nucleotides 694 to 741 of SEQ ID NO:1, nucleotides 961 to 1245 of SEQ ID NO:1, the complement of nucleotides 694 to 741 of SEQ ID NO:1, and the complement of nucleotides 961 to 1245 of SEQ ID NO:1.
7. The isolated nucleic acid molecule of claim 6, wherein any difference between the amino acid sequence encoded by the nucleic acid molecule and the corresponding amino acid sequence of SEQ ID NO:2 is due to a conservative amino acid substitution.
8. The isolated nucleic acid molecule of claim 6, comprising the nucleotide sequence of SEQ ID NO:1.
9. The isolated nucleic acid molecule of claim 6, wherein the nucleic acid molecule comprises a nucleotide sequence consisting of either nucleotides 694 to 741 of SEQ ID NO:1, or nucleotides 961 to 1245 of SEQ ID NO:1.
10. A vector, comprising the isolated nucleic acid molecule of claim 8.
11. An expression vector, comprising the isolated nucleic acid molecule of claim 8, a transcription promoter, and a transcription terminator, wherein the promoter is operably linked with the nucleic acid molecule, and wherein the nucleic acid molecule is operably linked with the transcription terminator.
12. A recombinant host cell comprising the expression vector of claim 11, wherein the host cell is selected from the group consisting of bacterium, yeast cell, avian cell, fungal cell, insect cell, mammalian cell, and plant cell.
13. A method of using the expression vector of claim 11 to produce Zwnt3 protein, comprising culturing recombinant host cells that comprise the expression vector and that produce the Zwnt3 protein.

14. The method of claim 13, further comprising isolating the *Zwnt3* protein from the cultured recombinant host cells.
15. An antibody or antibody fragment that specifically binds with the polypeptide of claim 3.
16. The antibody of claim 15, wherein the antibody is selected from the group consisting of: (a) polyclonal antibody, (b) murine monoclonal antibody, (c) humanized antibody derived from (b), and (d) human monoclonal antibody.
17. A method of detecting the presence of *Zwnt3* RNA in a biological sample, comprising:
 - (a) contacting a *Zwnt3* nucleic acid probe under hybridizing conditions with either (i) test RNA molecules isolated from the biological sample, or (ii) nucleic acid molecules synthesized from the isolated RNA molecules, wherein the probe consists of a nucleotide sequence comprising a portion of the nucleotide sequence of the nucleic acid molecule of claim 8, or complements thereof, and
 - (b) detecting the formation of hybrids of the nucleic acid probe and either the test RNA molecules or the synthesized nucleic acid molecules, wherein the presence of the hybrids indicates the presence of *Zwnt3* RNA in the biological sample.
18. A method of detecting the presence of *Zwnt3* in a biological sample, comprising:
 - (a) contacting the biological sample with an antibody, or an antibody fragment, of claim 15, wherein the contacting is performed under conditions that allow the binding of the antibody or antibody fragment to the biological sample, and
 - (b) detecting any of the bound antibody or bound antibody fragment.
19. An anti-idiotypic antibody, or anti-idiotypic antibody fragment, that specifically binds with the antibody or antibody fragment of claim 15.
20. A fusion protein, comprising the polypeptide of claim 3.

<400> 1

atg ggg aac ctg ttt atg ctc tgg gca gct ctg ggc ata tgc tgt gct 48
Met Gly Asn Leu Phe Met Leu Trp Ala Ala Leu Gly Ile Cys Cys Ala
1 5 10 15

gca ttc agt gcc tct gcc tgg tca gtg aac aat ttc ctg ata aca ggt 96
Ala Phe Ser Ala Ser Ala Trp Ser Val Asn Asn Phe Leu Ile Thr Gly
20 25 30

ccc aag gcc tat ctg acc tac acg act agt gtg gcc ttg ggt gcc cag 144
Pro Lys Ala Tyr Leu Thr Tyr Thr Ser Val Ala Leu Gly Ala Gln
35 40 45

agt ggc atc gag gag tgc aag ttc cag ttt gct tgg gaa cgc tgg aac 192
 Ser Gly Ile Glu Glu Cys Lys Phe Gln Phe Ala Trp Glu Arg Trp Asn
 50 55 60

tgc cct gaa aat gct ctt cag ctc tcc acc cac aac agg ctg aga agt 240
Cys Pro Glu Asn Ala Leu Gln Leu Ser Thr His Asn Arg Leu Arg Ser
65 70 75 80

gct acc aga gag act tcc ttc ata cat gct atc agc tct gct gga gtc Ala Thr Arg Glu Thr Ser Phe Ile His Ala Ile Ser Ser Ala Gly Val	288
85 90 95	
atg tac atc atc acc aag aac tgt agc atg ggt gac ttc gaa aac tgt Met Tyr Ile Ile Thr Lys Asn Cys Ser Met Gly Asp Phe Glu Asn Cys	336
100 105 110	
ggc tgt gat ggg tca aac aat gga aaa aca gga ggc cat ggc tgg atc Gly Cys Asp Gly Ser Asn Asn Gly Lys Thr Gly Gly His Gly Trp Ile	384
115 120 125	
tgg gga ggc tgc agc gac aat gtg gaa ttt ggg gaa agg atc tcc aaa Trp Gly Gly Cys Ser Asp Asn Val Glu Phe Gly Glu Arg Ile Ser Lys	432
130 135 140	
ctc ttt gtg gac agt ttg gag aag ggg aag gat gcc aga gcc ctg atg Leu Phe Val Asp Ser Leu Glu Lys Gly Lys Asp Ala Arg Ala Leu Met	480
145 150 155 160	
aat ctt cac aac aac agg gcc ggc aga ctg gtg gtg aga gcc acc atg Asn Leu His Asn Asn Arg Ala Gly Arg Leu Val Val Arg Ala Thr Met	528
165 170 175	
aaa agg aca tgc aaa tgt cat ggc atc tct ggg agc tgc agc ata cag Lys Arg Thr Cys Lys Cys His Gly Ile Ser Gly Ser Cys Ser Ile Gln	576
180 185 190	
aca tgc tgg ctg cag ctg gct gaa ttc cgg gag atg gga gac tac cta Thr Cys Trp Leu Gln Leu Ala Glu Phe Arg Glu Met Gly Asp Tyr Leu	624
195 200 205	
aag gcc aag tat gac cag gcg ctg aaa att gaa atg gat aag cgg cag Lys Ala Lys Tyr Asp Gln Ala Leu Lys Ile Glu Met Asp Lys Arg Gln	672
210 215 220	
ctg aga gct ggg aac agc gcc gag ggc cac tgg gtg ccc gct gag gcc Leu Arg Ala Gly Asn Ser Ala Glu Gly His Trp Val Pro Ala Glu Ala	720
225 230 235 240	
ttc ctt cct agc gca gag gcg gaa ctg atc ttt tta gag gaa tca cca Phe Leu Pro Ser Ala Glu Ala Glu Leu Ile Phe Leu Glu Glu Ser Pro	768
245 250 255	

gat tac tgt acc tgc aat tcc agc ctg ggc atc tat ggc aca gag ggt Asp Tyr Cys Thr Cys Asn Ser Ser Leu Gly Ile Tyr Gly Thr Glu Gly 260 265 270	816
cgt gag tgc cta cag aac agc cac aac aca tcc agg tgg gag cga cgt Arg Glu Cys Leu Gln Asn Ser His Asn Thr Ser Arg Trp Glu Arg Arg 275 280 285	864
agc tgt ggg cgc ctg tgc act gag tgt ggg ctg cag gtg gaa gag agg Ser Cys Gly Arg Leu Cys Thr Glu Cys Gly Leu Gln Val Glu Glu Arg 290 295 300	912
aaa act gag gtc ata agc agc tgt aac tgc aaa ttc cag tgg tgc tgt Lys Thr Glu Val Ile Ser Ser Cys Asn Cys Lys Phe Gln Trp Cys Cys 305 310 315 320	960
acg gtc aag tgt gac cag tgt agg cat gtg gtg agc aag tat tac tgc Thr Val Lys Cys Asp Gln Cys Arg His Val Val Ser Lys Tyr Tyr Cys 325 330 335	1008
gca cgc tcc cca ggc agt gcc cag tcc ctg gag cta tca gtc aca cca Ala Arg Ser Pro Gly Ser Ala Gln Ser Leu Glu Leu Ser Val Thr Pro 340 345 350	1056
acc aat ctt ccc aca tgg acc ctg tgc cag aaa caa cag gaa ttt gga Thr Asn Leu Pro Thr Trp Thr Leu Cys Gln Lys Gln Gln Glu Phe Gly 355 360 365	1104
ttt ctc tac atc cat cgc ctt cct gcc aag gat tca ttc caa ggc aac Phe Leu Tyr Ile His Arg Leu Pro Ala Lys Asp Ser Phe Gln Gly Asn 370 375 380	1152
aca gcc tca ttc aga ttt gtc agt tac agc cct ata tcc ctt ccc ttt Thr Ala Ser Phe Arg Phe Val Ser Tyr Ser Pro Ile Ser Leu Pro Phe 385 390 395 400	1200
tgg ttc atc ctt aac aag ctg gct atc att aag gtg aca gaa cag Trp Phe Ile Leu Asn Lys Leu Ala Ile Ile Lys Val Thr Glu Gln 405 410 415	1245

<210> 2

<211> 415

<212> PRT

<213> Homo sapiens

<400> 2

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 Pro Lys Ala Tyr Leu Thr Tyr Thr Thr Ser Val Ala Leu Gly Ala Gln
 35 40 45
 Ser Gly Ile Glu Glu Cys Lys Phe Gln Phe Ala Trp Glu Arg Trp Asn
 50 55 60
 Cys Pro Glu Asn Ala Leu Gln Leu Ser Thr His Asn Arg Leu Arg Ser
 65 70 75 80
 Ala Thr Arg Glu Thr Ser Phe Ile His Ala Ile Ser Ser Ala Gly Val
 85 90 95
 Met Tyr Ile Ile Thr Lys Asn Cys Ser Met Gly Asp Phe Glu Asn Cys
 100 105 110
 Gly Cys Asp Gly Ser Asn Asn Gly Lys Thr Gly Gly His Gly Trp Ile
 115 120 125
 Trp Gly Gly Cys Ser Asp Asn Val Glu Phe Gly Glu Arg Ile Ser Lys
 130 135 140
 Leu Phe Val Asp Ser Leu Glu Lys Gly Lys Asp Ala Arg Ala Leu Met
 145 150 155 160
 Asn Leu His Asn Asn Arg Ala Gly Arg Leu Ala Val Arg Ala Thr Met
 165 170 175
 Lys Arg Thr Cys Lys Cys His Gly Ile Ser Gly Ser Cys Ser Ile Gln
 180 185 190
 Thr Cys Trp Leu Gln Leu Ala Glu Phe Arg Glu Met Gly Asp Tyr Leu
 195 200 205
 Lys Ala Lys Tyr Asp Gln Ala Leu Lys Ile Glu Met Asp Lys Arg Gln
 210 215 220
 Leu Arg Ala Gly Asn Ser Ala Glu Gly His Trp Val Pro Ala Glu Ala
 225 230 235 240
 Phe Leu Pro Ser Ala Glu Ala Glu Leu Ile Phe Leu Glu Glu Ser Pro
 245 250 255
 Asp Tyr Cys Thr Cys Asn Ser Ser Leu Gly Ile Tyr Gly Thr Glu Gly
 260 265 270
 Arg Glu Cys Leu Gln Asn Ser His Asn Thr Ser Arg Trp Glu Arg Arg
 275 280 285
 Ser Cys Gly Arg Leu Cys Thr Glu Cys Gly Leu Gln Val Glu Glu Arg
 290 295 300
 Lys Thr Glu Val Ile Ser Ser Cys Asn Cys Lys Phe Gln Trp Cys Cys
 305 310 315 320

Thr Val Lys Cys Asp Gln Cys Arg His Val Val Ser Lys Tyr Tyr Cys
 325 330 335
 Ala Arg Ser Pro Gly Ser Ala Gln Ser Leu Glu Leu Ser Val Thr Pro
 340 345 350
 Thr Asn Leu Pro Thr Trp Thr Leu Cys Gln Lys Gln Gln Glu Phe Gly
 355 360 365
 Phe Leu Tyr Ile His Arg Leu Pro Ala Lys Asp Ser Phe Gln Gly Asn
 370 375 380
 Thr Ala Ser Phe Arg Phe Val Ser Tyr Ser Pro Ile Ser Leu Pro Phe
 385 390 395 400
 Trp Phe Ile Leu Asn Lys Leu Ala Ile Ile Lys Val Thr Glu Gln
 405 410 415

<210> 3

<211> 1245

<212> DNA

<213> Artificial Sequence

<220>

<223> This degenerate sequence encodes the amino acid
sequence of SEQ ID NO:2.

<221> variation

<222> (1)...(1245)

<223> N is any nucleotide.

<400> 3

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wsngcntggw sngtnaayaa ytttytnath acnggnccna argcntayyt nacntayacn	120
acnwsngtng cnytnngngc ncarwsnggn athgargart gyaarttyca rttygcntgg	180
garngntgga aytgyccnga raaygcnytn carytnwsna cncayaaymg nytnmgwnsn	240
gcnacnmng arcnwsntt yathcaygc athwsnwsng cngngtnat gtayathath	300
acnaaraayt gywsnatggg ngayttygar aaytgyggnt gygayggngws naayaaygg	360
aaracngng gncayggntg gathtgggn ggntgywsng ayaaygtnga rttyggngar	420
mgnathwsna arytnntygt ngaywsnytn garaaggna argaygcnmg ngcnytnatg	480
aayytncaya ayaaymgngc nggnmgnytn gcngtnmgng cnacnatgaa rmgnacntgy	540
aartgycaay gnathwsngg nwsntgywsn athcaracnt gytggytnca rytngcngar	600
ttymgngara tggngayta yytnaargcn aartaygayc argcnytnaa rathgaratg	660
gayaarmgnc arytnmgngc nggnaaywsn gcngarggnc aytgggtnc ngcngargcn	720
ttytnccnw sngcngargc ngarytnath ttyytnarg arwsnccnga ytaytgyacn	780
tgyaaywsnw snytnngnat htaygggnacn garggnmgng artgyytnc raaywsncay	840
aayacnwsnm gntgggarmg nmgnwsntgy ggnmgnytn gyacngartg yggnytnear	900
gtngargarm gnaaracnga rgtathwsn wsntgyaayt gyaarttyca rtggtgytgy	960

acngtnaart gygaycartg ymgncaygtn gtnwsnaart aytaytgygc nmgnwsnccn 1020
 ggnwsngcnc arwsnytn ga rytnwsngtn acnccnacna ayytnccnac ntggacnytn 1080
 tgycaraarc arcargartt yggnttyytn tayathcaym gnytnccngc naargaywsn 1140
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 tggtyathy tnaayaaryt ngcnathath aargtnacng arcar 1245

<210> 4
 <211> 354
 <212> PRT
 <213> Mouse

<400> 4

Met Gly His Leu Leu Met Leu Trp Val Ala Ala Gly Met Cys Tyr Pro
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 Ala Leu Gly Ala Ser Ala Trp Ser Val Asn Asn Phe Leu Ile Thr Gly
 20 25 30
 Pro Lys Ala Tyr Leu Thr Tyr Thr Ala Ser Val Ala Leu Gly Ala Gln
 35 40 45
 Ile Gly Ile Glu Glu Cys Lys Phe Gln Phe Ala Trp Glu Arg Trp Asn
 50 55 60
 Cys Pro Glu His Ala Phe Gln Phe Ser Thr His Asn Arg Leu Arg Ala
 65 70 75 80
 Ala Thr Arg Glu Thr Ser Phe Ile His Ala Ile Arg Ser Ala Ala Ile
 85 90 95
 Met Tyr Ala Val Thr Lys Asn Cys Ser Met Gly Asp Leu Glu Asn Cys
 100 105 110
 Gly Cys Asp Glu Ser Gln Asn Gly Lys Thr Gly Gly His Gly Trp Ile
 115 120 125
 Trp Gly Gly Cys Ser Asp Asn Val Glu Phe Gly Glu Lys Ile Ser Arg
 130 135 140
 Leu Phe Val Asp Ser Leu Glu Lys Gly Lys Asp Ala Arg Ala Leu Val
 145 150 155 160
 Asn Leu His Asn Asn Arg Ala Gly Arg Leu Ala Val Arg Ala Ser Thr
 165 170 175
 Lys Arg Thr Cys Lys Cys His Gly Ile Ser Gly Ser Cys Ser Ile Gln
 180 185 190
 Thr Cys Trp Leu Gln Leu Ala Asp Phe Arg Gln Met Gly Asn Tyr Leu
 195 200 205
 Lys Ala Lys Tyr Asp Arg Ala Leu Lys Ile Glu Met Asp Lys Arg Gln
 210 215 220
 Leu Arg Ala Gly Asn Arg Ala Glu Gly Arg Trp Ala Leu Thr Glu Ala
 225 230 235 240

Phe Leu Pro Ser Thr Glu Ala Glu Leu Ile Phe Leu Glu Gly Ser Pro
 245 250 255
 Asp Tyr Cys Asn Arg Asn Ala Ser Leu Ser Ile Gln Gly Thr Glu Gly
 260 265 270
 Arg Glu Cys Leu Gln Asn Ala Arg Ser Ala Ser Arg Arg Glu Gln Arg
 275 280 285
 Ser Cys Gly Arg Leu Cys Thr Glu Cys Gly Leu Gln Val Glu Glu Arg
 290 295 300
 Arg Ala Glu Ala Val Ser Ser Cys Asp Cys Asn Phe Gln Trp Cys Cys
 305 310 315 320
 Thr Val Lys Cys Gly Gln Cys Arg Arg Val Val Ser Arg Tyr Tyr Cys
 325 330 335
 Thr Arg Pro Val Gly Ser Ala Arg Pro Arg Gly Arg Gly Lys Asp Ser
 340 345 350
 Ala Trp

<210> 5
 <211> 16
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Peptide linker.

<400> 5
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 1 5 10 15

<210> 6
 <211> 295
 <212> PRT
 <213> Homo sapiens

<400> 6
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 Tyr Ser Ser Ser Val Ala Ala Gly Ala Gln Ser Gly Ile Glu Glu Cys
 20 25 30
 Lys Tyr Gln Phe Ala Trp Asp Arg Trp Asn Cys Pro Glu Arg Ala Leu
 35 40 45
 Gln Leu Ser Ser His Gly Gly Leu Arg Ser Ala Asn Arg Glu Thr Ala
 50 55 60

Phe Val His Ala Ile Ser Ser Ala Gly Val Met Tyr Thr Leu Thr Arg
 65 70 75 80
 Asn Cys Ser Leu Gly Asp Phe Asp Asn Cys Gly Cys Asp Asp Ser Arg
 85 90 95
 Asn Gly Gln Leu Gly Gly Gln Gly Trp Leu Trp Gly Gly Cys Ser Asp
 100 105 110
 Asn Val Gly Phe Gly Glu Ala Ile Ser Lys Gln Phe Val Asp Ala Leu
 115 120 125
 Glu Thr Gly Gln Asp Ala Arg Ala Ala Met Asn Leu His Asn Asn Glu
 130 135 140
 Ala Gly Arg Lys Ala Val Lys Gly Thr Met Lys Arg Thr Cys Lys Cys
 145 150 155 160
 His Gly Val Ser Gly Ser Cys Thr Thr Gln Thr Cys Trp Leu Gln Leu
 165 170 175
 Pro Glu Phe Arg Glu Val Gly Ala His Leu Lys Glu Lys Tyr His Ala
 180 185 190
 Ala Leu Lys Val Asp Leu Leu Gln Gly Ala Gly Asn Ser Ala Ala Ala
 195 200 205
 Arg Gly Ala Ile Ala Asp Thr Phe Arg Ser Ile Ser Thr Arg Glu Leu
 210 215 220
 Val His Leu Glu Asp Ser Pro Asp Tyr Cys Leu Glu Asn Lys Thr Leu
 225 230 235 240
 Gly Leu Leu Gly Thr Glu Gly Arg Glu Cys Leu Arg Arg Gly Arg Ala
 245 250 255
 Leu Gly Arg Trp Glu Leu Arg Ser Cys Arg Arg Leu Cys Gly Asp Cys
 260 265 270
 Gly Leu Ala Val Glu Glu Arg Arg Ala Glu Thr Val Ser Ser Cys Asn
 275 280 285
 Cys Lys Phe His Trp Cys Cys
 290 295